The roles of Off-track and Semaphorin-1a in *Drosophila* visual system development

by

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List of abbreviations used

AB	Antibody
Beat	Beaten path
Bks	Brakeless
cAMP	Cyclic adenosine monophosphate
CCK-4	Colon carcinoma kinase-4
CNS	Central nervous system
CRIB	Cdc42/Rac interactive binding
C-terminal	Carboxyl-terminal
DAB	Diaminobenzidene
DSHB	Developmental studies hybridoma bank
DInR	*Drosophila* insulin receptor
Dock	Dreadlocks
Drl	Derailed
EGFR	Epidermal growth factor receptor
EMS	Ethanemethanesulfonate
Ena	Enabled
ErbB	Erythroblastosis B
F-actin	Filamentous actin
FAS	Fasciclin
FLP	FLIP recombinase
FMI	Flamingo
FN	Fibronectin
FRT	FLP recombinase target
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescence protein
GOF	Gain-of-function
GST	Glutathionine *S*-transferase
Ig	Immunoglobulin
ISN	Intersegmental nerve
LAR	Leukocyte antigen-related

LOF	Loss-of-function
MARCM	Mosaic analysis with a repressible cell marker
MICAL	Molecule interacting with CasL
Msn	Misshapen
NGS	Normal goat serum
N-terminal	Amino-terminal
OCT	Optimal cutting temperature
Otk	Off-track
Pak	p21-activated kinase
PBS	Phosphate-buffered saline
PKA	Protein kinase A
PlexA	Plexin A
PTP	Protein tyrosine phosphatase
R-cell	Photoreceptor cell
Run	Runt
Sbb	Scribbler
Sema	Semaphorin
Sdk	Sidekick
SH	Src-homology
SN	Segmental nerve
Til	Tibia 1
UV/vis	Ultraviolet/visual light
WT	Wild-type
Y 2-H	Yeast two-hybrid

Abstract

My PhD work focuses on the study of the layer-specific targeting of photoreceptor (R-cell) axons in the developing *Drosophila* visual system. More specifically, I have studied the roles of two molecules, the receptor tyrosine kinase Off-track (Otk) and the transmembrane protein Semaphorin1a (Sema1a) in R-cell axon guidance and target selection.

The *Drosophila* adult visual system is comprised of the compound eye and the optic lobe. The compound eye is comprised of ~800 ommatidia, each containing eight different R-cells. As the visual system develops, R-cells project axons through the optic stalk toward two different layers of the optic lobe. R1-6 axons innervate the superficial lamina layer while R7 and R8 axons project through the lamina into the deeper medulla layer. Previous studies demonstrated that the SH2/SH3 adapter protein Dock and two receptor protein tyrosine phosphatases LAR and PTP69D are required for the termination of R1-6 axons in the lamina, indicating that protein tyrosine phosphorylation is a key signaling event in regulating this process. However, nothing was known about the molecular nature of protein tyrosine kinases involved. By taking a testing-candidate-gene approach, I identified Otk as a key player in regulating R1-R6 axon targeting. Otk protein is expressed in R-cell axons and is predominantly localized to R1-6 growth cones. Phenotypic analysis of *otk* mutants using a set of developmental markers indicates that *otk* is specifically required for R1-6 growth-cone targeting. That R1-6 targeting defect in *otk* mutants could be rescued substantially by eye-specific expression of an *otk* transgene, together with the results from genetic mosaic analysis, indicates that *otk* is required in R1-6 growth cones for the lamina-specific termination. I propose that Otk functions as a receptor for a target-derived stop signal to terminate R1-R6 growth cones in the lamina.

Previous studies had implicated Sema1a as an upstream activator of Otk in *Drosophila* embryonic motor axon guidance. I compared the loss-of-function phenotypes of both *sema1a* and *otk* in order to test whether *sema1a* may also function upstream of *otk* in the visual system. We found that loss of *sema1a* caused an R-cell projection phenotype different from that in *otk* mutants, suggesting that *sema1a* plays a different role in R-cell axon guidance. Our immunohistological analysis demonstrated that Sema1a is present on R-cell axons and growth cones. Loss-of-function analysis reveals a requirement for *sema1a* in the association of R1-R6 growth cones in the lamina. Consistently, overexpression of *sema1a* caused the formation of abnormal thicker bundles in a dosage-dependent manner. Interestingly, we found that the cytoplasmic domain of Sema1a is absolutely required for its function in R-cell axon guidance, suggesting a model in which Sema1a functions as a guidance receptor to mediate R-cell growth-cone interactions during R-cell axon projections.

Résumé

Mon doctorat porte sur l'étude des mécanismes impliqués dans le guidage d'axones des photorécepteurs (cellules R) vers des couches spécifiques du système visuel de la *Drosophile* en développement. Plus précisément, j'ai étudié le rôle de deux molécules, le récepteur tyrosine kinase Off-track (Otk) et la protéine transmembranaire Semaphorin1a (Sema1a), dans les processus de guidage des axons de cellules R vers des couches de tissus cibles.

Le système visuel de la *Drosophile* adulte est composé de l'œil externe et du lobe optique. L'œil externe est composé de ~800 ommatidies, chacune étant comprises de huit différentes cellules R. Au cours du développement du système visuel, les cellules R projettent leur axone à travers le pédicule optique vers deux différentes couches du lobe optique. Les axones R1-6 innervent la couche superficielle lamellaire alors que les axones R7 et R8 sont projetés à travers la couche lamellaire et innervent la couche médullaire plus profonde. D'autres études ont démontré que la protéine SH2/SH3 adaptatrice Dock et les deux protéines récepteurs tyrosine phosphatases Dlar et DPTP69D sont nécessaires à la localisation des axones R1-6 dans la couche lamellaire, indiquant que la phosphorylation de résidus protéiques de tyrosine est une étape clé dans la régulation de ce processus. Cependant, aucune évidence concernant la nature moléculaire des tyrosine kinases impliquées dans ce processus n'était jusqu'à ce jour disponible. En utilisant une approche test-candidat-gène, j'ai pu identifier Otk comme étant un contributeur important dans le guidage des axones R1-6 vers leur couche cible. La protéine Otk est exprimée dans les axones des cellules R avec une prépondérance dans les cônes de croissance des cellules R1-6. En utilisant différents marqueurs du développement, l'analyse phénotypique de mutants *otk* indique que la protéine *otk* est essentielle au guidage des cônes de croissance R1-6. L'incapacité des mutants *otk* à guider correctement les cellules R1-6 ayant pu être réchappée par l'expression spécifique d'un transgène *otk*, ainsi que les résultats de l'analyse d'une mosaïque génétique, indiquent que *otk* est nécessaire à la terminaison et à la localisation spécifique des cônes de croissance R1-6 dans la couche lamellaire du lobe optique. Je propose donc que Otk fonctionne comme le récepteur d'un signal provenant de la couche cible et servant à arrêter la progression des cônes de croissance des cellules R1-6 dans la couche lamellaire.

D'autres études ont impliqué Sema1a comme un activateur en amont de la protéine Otk, elle-même engagée dans le guidage d'axones moteurs chez la *Drosophile* durant le stade embryonal. J'ai comparé les phénotypes de perte de fonction de *sema1a* et *otk* dans le but de vérifier si *sema1a* fonctionne aussi comme un activateur d'*otk* dans le système visuel. Les mutants *sema1a* présentent une projection des cellules R différente de celle des mutants *otk*, ce qui suggère que *sema1a* joue un rôle différent dans le guidage des cellules R. Nos analyses immunohistologiques démontrent bien que *sema1a* est présente dans les axones des cellules R ainsi que dans les cônes de croissance. Les analyses de perte de fonction révèlent la nécessité de *sema1a* dans l'association des cônes de croissance dans la couche lamellaire. De la même manière, la surexpression de *sema1a* cause la formation d'épais faisceaux d'axones anormaux variant selon le niveau d'expression. Nous avons aussi découvert que le domaine cytoplasmique de *sema1a* est absolument nécessaire à sa fonction dans le guidage des axones de cellules R. Cela suggère un modèle dans lequel Sema1a fonctionne comme un récepteur de guidage qui assure l'interaction des cônes de croissance de cellules R durant la projection de leur axones.

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Contributions of co-authors

In Chapter 2, **Li Yu** helped to quantify the numbers of axonal bundles found in the medulla of *otk* mutants, stained glial cells in *otk* mutants, and examined *sema1a*^{P1} mosaics labeled with *rough-τ-lacZ.*

In Chapter 3, **Li Yu** examined *sema1a*^{P1}*/Df(2)N22-5* hemizygotes, generated single *sema1a* mutant R-cell axons using the MARCM technique, removed *MICAL* dosage in a *sema1a* GOF background, overexpressed *sema1a*$^{\Delta cyt}$ in the eye, and assembled table 1 entitled "Quantification of the axonal hyper-fasciculation phenotype in third-instar larvae."

Dr. Yong Rao is the primary investigator and research supervisor for Li Yu and Patrick Cafferty.

Original contributions to knowledge

Chapter 2: The data presented in this chapter report the first receptor tyrosine kinase involved in the layer-specific targeting of R1-R6 axons in the *Drosophila* visual system. While tyrosine phosphorylation was known to play a key signaling role in R-cell growth cone targeting due to the requirement of the SH2/SH3 domain-containing molecule, Dock, and the two receptor tyrosine phosphatases, PTP69D and LAR, Otk is the first receptor tyrosine kinase identified in this process. Additionally, we provide evidence that suggests Otk is the first cell-surface receptor to act in an instructive manner in the targeting of R1-R6 axons in the visual system.

Chapter 3: Sema1a was previously shown in *Drosophila* to act as an Otk-ligand to regulate the defasciculation of motor axons. In this chapter we provide the first evidence that Sema1a can function as an attractive receptor in axon guidance.

Chapter 1

Introduction and literature review

1.1 Introduction to the field of axonal guidance and targeting

1.1.1 The role of the growth cone

The daunting task of understanding the processes required to build a functional nervous system began over 100 years ago with examinations of vertebrate species, though over time use of invertebrate systems, including studies of grasshopper embryo development and modern genetic analysis with *Drosophila* and *Caenorhabditis elegans*, have yielded a wealth of valuable information (VanVactor and Lorenz, 1999). The proper development of vertebrate and invertebrate nervous systems requires the formation of specific connections between neurons and their synaptic targets (Goodman, 1996). The distance between the post-mitotic neuron and the target can be very large, as much as several centimeters (Tessier-Lavigne and Goodman, 1996). The process of connection formation is directed by the interaction of the growth cone, a structure located at the leading edge of the axon sent by a neuron, with the surrounding environment (Goodman, 1996). Growth cones are composed of two regions: an actin-rich peripheral region composed of filopodia and lamellipodia and a microtubule-rich central region (Dent and Gertler, 2003). Extracellular guidance cues are detected by cell-surface receptors on the growth cone and signal transduction mechanisms translate these guidance signals to direct the rate and direction of growth of the underlying cytoskeletal elements within the growth cone.

1.1.2 GTPases regulate cytoskeletal changes in the growth cone

The polymerization of actin into filaments within the growth cone is regulated by Rho GTPases (Mueller, 1999). GTPases cycle between active GTP-bound states and, following hydrolysis of GTP to GDP and orthophosphate, inactive GDP-bound states. The replacement of a GDP with a GTP molecule results in GTPase activation (Hall, 1994). Studies *in vitro* have revealed the abilities of three Rho GTPases, Cdc42, Rac, and Rho to regulate the formation of different actin structures. Injection of active Cdc42 (Kozma *et al.*, 1995), Rac (Ridley *et al.*, 1992), and Rho (Ridley and Hall, 1992) into fibroblast cells has been demonstrated to induce the formation of finger-like protrusions called filopodia, fan-shaped membrane extensions called lamelliopdia, and cell-substrate anchoring focal adhesions and stress fibers, respectively. These *in vitro* results suggest that attractive guidance cues may activate Rac or Cdc42 while repulsive cues may activate Rho in the growth cone and recent *in vivo* data has provided further support for this model. Hu *et al.* (2001) have demonstrated genetically that Plexin B, a growth cone receptor, acts to inhibit Rac and activate Rho in the repulsion of motoneurons in the *Drosophila* embryo while an *in vitro* competition assay revealed that Plexin B can compete for Rac-binding with p21-activated kinase (Pak), a downstream effector of Rac. Additionally, Vikis *et al.* (2002) have demonstrated that overexpression of the cytoplasmic domain of Plexin B1 in mammalian HEK293 cells can inhibit Pak activation.

Rho GTPases are regulated by a number of upstream effectors including guanine nucleotide exchange factors (GEFs) that stimulate the exchange of GTP for GDP by GTPases, GTPase-activating proteins (GAPs) that promote GTPase activity, and guanine nucleotide dissociation inhibitors (GDIs) that stabilize GTPases in the GDP-bound state

(Mueller, 1999). Newsome *et al.* (2000) have demonstrated a requirement for a GEF called Trio for the proper guidance of photoreceptor (R-cell) axons in the *Drosophila* visual system. The roles of Trio and Rac in R-cell axon guidance will be described in further detail in section 1.2.3.5 of this thesis.

1.1.3 Pioneer axons and labeled pathways

The routes traveled by an extending axon may be subdivided into smaller and distinct segments to aid the pathfinding of a growth cone toward a final target cell. For example, the tibia 1 (Ti1) neuron of the grasshopper embryo must grow from the developing leg into the central nervous system (CNS) using specialized "guidepost cells," in order to navigate the turns required to physically reach the target (Caudy and Bentley, 1986). Selective destruction of "guidepost cells" resulted in the growth of Ti1 astray from the target.

The basic manner with which the wiring of the nervous system is thought to occur was first described in 1910 (Harrison, 1910). Harrison (1910) noted that pioneer axons of the amphibian peripheral nervous system extended toward target cells early in development when the distance from cell body to target is relatively short. Axons that must reach target cells at later developmental stages extend across longer distances by following the paths of, or fasciculating along, the surfaces of the original pioneers. The underlying mechanisms of axonal fasciculation not understood by Harrison have since been greatly studied. The observation that G and C axons in the grasshopper embryo regularly fasciculate along the paths of specific earlier differentiated axons, despite having already traversed the paths of many other neuronal cells led to the proposal of the "labeled pathways" theory (Raper *et al.*, 1983). According to this idea, particular axons

would be labeled with different cell surface molecules that could be used to guide specific growth cones in the appropriate direction. The first pathway labels, called Fasciclin (Fas) I and II were identified raising monoclonal antibodies against axonal fascicle cell-surface antigens and immunoprecipitating molecules from the grasshopper CNS (Bastiani *et al.*, 1987). Further identification and characterization of molecules involved in invertebrate axonal fasciculation was accomplished using the *Drosophila* embryonic CNS and motor nervous system as a model.

Goodman *et al.* (1984) found that the "*Drosophila* embryo CNS is a miniature replica of the grasshopper embryo in terms of its identified neurons, their growth cones, and their selective fasciculation choices." The similarities of the previously studied grasshopper and *Drosophila* embryos (Goodman *et al.*, 1984) prompted many researchers to study axonal development using *Drosophila* as a model, in the hopes that a molecular genetic approach could be adopted for the study of molecules involved in axonal pathfinding and targeting. Indeed, the loss-of-function (LOF) and gain-of-function (GOF) phenotypes of Fas II have been well characterized in *Drosophila* (Grenningloh *et al.*, 1991; Lin and Goodman, 1994) and these findings will be further discussed in section 1.3.1 of this introduction. While the pathfinding of secondary afferents is simplified by adherence to pioneer axons through cell-adhesion molecules such as Fas II, ultimately these secondary afferents must release from the pioneer axon in order to properly innervate target cells. One molecule demonstrated as being involved in the facilitation of motor axon defasciculation at specific choice points is the transmembrane Semaphorin (Sema) Sema1a (Yu *et al.*, 1998). Sema1a has been shown to have an antagonistic effect on the adherence mediated by Fas II (Yu *et al.*, 2000). In chapter 3 of this thesis, I will

present evidence that suggests Sema1a can also act to promote axonal fasciculation in the developing *Drosophila* visual system.

1.1.4 An introduction to Semaphorin proteins

The Semaphorins (Sema) are a family of both secreted and cell-surface molecules that all share a conserved 500 amino acid amino-terminal "Sema" domain (reviewed in Nakamura *et al.*, 2000; Tamagnone and Comoglio, 2000; Castellani and Rougon, 2002; Pasterkamp and Kolodkin, 2003). Sema proteins are involved in a large number of biological processes, including axon guidance, lymphocyte activation, vascular endothelial cell motility, and lung branching morphogenesis (Goshima *et al.*, 2002). The Sema proteins have been grouped into 8 classes: classes 1 and 2 are found in invertebrates; classes 3-7 are found in vertebrates; and the final group is found in viruses (Kruger *et al.*, 2005). The Sema proteins found in classes 2 and 3 and the viral encoded Sema are all secreted molecules while Sema proteins found in all other classes are membrane bound. The role of Sema in axonal guidance has been described as that of an upstream ligand of Plexin proteins in invertebrates or, in the case of class A Plexin proteins, both Plexin and the Neuropilin co-receptor, in order to mediate a repulsive axonal response (Kruger *et al.*, 2005). Sema3A was the first Sema protein identified in vertebrates (Luo *et al.*, 1993).

Luo *et al.* (1993) had demonstrated that Sema3A could induce growth cone collapse of dorsal root ganglion axons *in vitro*. Subsequent work has further established the repulsive action of Sema3A *in vivo*. For example, Huber *et al.* (2005) have shown that Sema3A is expressed in the developing mouse forelimb and is required for proper limb innervation by motor axons. Normally, vertebrate motor axons that grow from the

spinal cord to innervate the limb will pause at a plexus region prior to extension within the limb, as demonstrated in the chick by Wang and Scott (2000). This resting period may allow for the expression or organization of the signaling molecules required for proper targeting in the growth cone, within the target region, or both. In *sema3A* mutant mice motor axons grow prematurely into forelimb tissue and terminate in aberrant regions (Huber *et al.*, 2005). Invertebrate Sema proteins have also been demonstrated as acting as repulsive guidance molecules. The role of Sema1a as a repulsive for motor axons in the *Drosophila* motor nervous system will be described in detail in section 1.3.3 of this thesis.

1.1.5 Growth cone response can vary depending upon the cyclic nucleotide levels in the growth cone

A large body of *in vitro* evidence supports the idea that cyclic nucleotide levels within the growth cone can determine whether the growth cone interprets a cue as repulsive or attractive. Song and Poo (1999) have demonstrated using cultured *Xenopus* spinal neurons in an *in vitro* turning assay that an axon can respond differently to a cue depending upon intracellular cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophospate (cGMP) levels. *In vitro*, Ming *et al.* (1997) have shown a conversion from an attractive to a repulsive response by a growth cone to Netrin-1 by decreasing cAMP concentration in the growth cone while Song *et al.* (1998) have altered the repulsive response of a growth cone to Sema3A by increasing cGMP levels within the growth cone. Dontchev and Letourneau (2002) have provided evidence that Sema mediated responses can be dependant upon the ratio of cAMP to cGMP concentrations: a high level of cAMP to cGMP concentration led to a failure of growth cones to collapse in response to Sema3A while a low level of cAMP to cGMP led to growth cone collapse.

Recent *in vivo* evidence supports *in vitro* data that suggests growth cone cyclic nucleotide levels are important to determine the direction of growth cone response. Using a biochemical (Terman and Kolodkin, 2004) and a genetic screen (Ayoob *et al.*, 2004), Kolodkin and colleagues have identified two molecules involved in regulating cyclic nucleotide levels in the growth cones of *Drosophila* motor axons. Terman and Kolodkin (2004) have demonstrated that the A kinase anchoring protein Nervy and protein kinase A (PKA) can antagonize Sema1a signaling in the motor nervous system, while Ayoob *et al.* (2004) have shown that the receptor Guanylyl Cyclase Gyc76C is necessary for repulsive Sema1a signaling. Together, these results indicate that the internal state of the growth cone is important to determine the response of the growth cone to a guidance cue.

1.1.6 Layer specific target selection

The vertebrate brain is organized into parallel layers or laminae composed of neurons "with similar functional properties and similar sets of connections" (Bolz and Castellani, 1997). The use of "stripe assays" *in vitro* has demonstrated the ability of axon afferents in culture to extend upon the same cortical layers of tissue explants as layers that are normally targeted *in vivo* (Bolz and Castellani, 1997). These "stripe assay" experiments indicate that layer selection of axon afferents is due to the intrinsic properties of specific lamina layers—that is, the selection of specific layers in the vertebrate brain is due to the interpretation of differentially expressed layer-specific cues by cell-surface receptors located on axon afferent growth cones (Bolz and Castellani, 1997). A number of molecules have been identified that are involved in vertebrate layer-

specific axonal targeting including N-Cadherin, Sidekick (Sdk) 1 and Sdk2, and ephrin-A5.

Antibody perturbation experiments have revealed the requirement for N-Cadherin in layer-specific selection of retinal axons that innervate the chick optic tectum (Inoue and Sanes, 1997). In the presence of a monoclonal anti-N-Cadherin antibody, axonal afferents bypass normal target layers. The immunoglobulin superfamily members Sidekick (Sdk) 1 and Sdk 2 have been demonstrated to mediate layer-specific selection in the chick optic tectum by retinal axons (Yamagata *et al.*, 2003). Yamagata *et al.* (2003) demonstrated that ectopic expression of Sdk-1 in retinal Sdk-1 negative cells diverted the growth of axons to Sdk-1 positive lamina layers. Members of the largest subgroup of receptor tyrosine kinases, Eph receptors and co-responding ephrin ligands (Flanagan and Vanderhaeghan, 1998), have also been implicated in vertebrate layer-specific axonal targeting (Mann *et al.*, 2002). Mann *et al.* (2002) observed an inability of thalamic axons to correctly recognize their normal target layer following the enzymatic removal of the GPI-anchored ligand ephrin-A5 from cortical membranes. The use of a simple model system, such as the *Drosophila* visual system, can facilitate the identification of further factors involved in layer-specific axonal targeting. In chapter 2 of this thesis, I will describe the role of the receptor tyrosine kinase Off-track (Otk) in the layer-specific targeting of axons in the *Drosophila* visual system.

1.1.7 Axon guidance and targeting in the vertebrate visual system

Studies of the vertebrate visual system have led to the identification of a large number of molecules required for proper optic axon guidance. As the vertebrate visual system develops retinal ganglion cells (RGC) extend axons toward the optic disc to the

optic stalk and form an optic chiasm where axons cross the midline of the diencephalon in a contralateral direction, though a small number of axons in vertebrates with binocular vision remain ipsilateral to the midline. Following further extension from the optic chiasm, final axonal connections are established with target neurons in the superior colliculus and the optic tectum (Inatani, 2005). The axons of younger RGC cells have been found to fasciculate along older pioneer RGC axons as they extend from the retina toward the optic disc. Bastmeyer *et al.* (1995) have demonstrated that this fasciculation process is dependent upon the L1 cell adhesion molecule as an anti-L1 antibody perturbation experiment revealed a disruption of retinal axon pathfinding. The extension of RGC axons toward the optic disc is promoted by the interaction of the secreted guidance cue, Netrin-1, expressed by glial cells in the eye disc, and the Deleted in Colorectal Cancer (DCC) receptor, expressed by RGC axons Deiner *et al.* (1997). Deiner *et al.* (1997) showed using an *in vitro* assay that retinal axon outgrowth is promoted by Netrin-1, but blocked by an anti-DCC antibody. The decision of whether RGC axons cross the optic chiasm midline to project toward the contralateral brain, or to continue extension through the optic chiasm and project into the ipsilateral brain is mediated by Ephrin ligands and Eph receptors (Flanagan and Vanderhaeghen, 1998).

Nakagawa *et al.* (2000) have demonstrated that Ephrin-B expression is required for the ipsilateral projection of a subset of RGC axons across the optic chiasm in *Xenopus* adults. During the *Xenopus* tadpole stage all RGC axons cross the optic chiasm in a contralateral direction. Nakagawa *et al.* (2000) observed that Ephrin-B expression is upregulated during metamorphosis. The binding of inhibitory Ephrin-B ligand to EphB1 receptors expressed by RGC axons likely prevents these axons from crossing the chiasm

in a contralateral fashion resulting in ipsilateral growth (Nakagawa, 2000). Similarly, Ephrin ligands and Eph receptors have also been demonstrated as necessary for proper topographic mapping of RCG axons, that is Ephrin ligand and Eph receptors are required for axons from neighbouring eye RCG to project to neighbouring regions in the brain. Topographic mapping of RCG axons results from the gradient distribution of specific Eph receptors expressed by retinal cells, and repulsive Ephrin ligands expressed in the superior colliculus/optic tectum (Flanagan and Vanderhaeghen, 1998). As an example, Ephrin-A2 and Ephrin-A5 repulse axons from temporal-region retinal axons from chick explants, but not axons from nasal-region explants (Monschau *et al.*, 1997). The result of a rostral-caudal expression gradient of Ephrin-A2 in the chick RGC axon target region, or of Ephrin-A5 in the mouse RGC axon target region, is that only RGC axons from the retinal nasal-region but not the temporal-region can extend into the posterior-most region of highest Ephrin-A2 or A5 expression (Flanagan and Vanderhaeghen, 1998). In this thesis, I will describe molecules required for proper axonal guidance and targeting of photoreceptor axons in the *Drosophila* visual system.

1.1.8 Development of the *Drosophila* visual system

The *Drosophila* visual system includes the compound eye and the optic lobe of the brain. The adult compound eye is made up of approximately 800 units, or ommatidia (Tomlinson and Ready, 1987a). Each ommatidium is composed of eight photoreceptor-cells (R-cells), and twelve accessory cells. The R-cells are elongated sensory neurons that contain rhabdomeres, photosensitive stacks of rhodopsin-bearing microvilli (Ready, 1989). The outer R1-R6 R-cells, that detect green light, surround both the R8 R-cell that responds to blue light, and the UV-light responsive R7 R-cell (Meinertzhagen and

Hanson, 1993). Late during the third, "wandering" larval stage and early during the pupal stages of *Drosophila* development, the R-cells, that total over 6000, project their axons along stereotyped paths from the eye imaginal disc, through the optic stalk, and into distinct neuronal layers, called the lamina and the medulla of the optic lobe of the brain. The R-cells form a topographic map within the optic lobe (Meinertzhagen and Hanson, 1993).

The first R-cells to differentiate are R8, followed by R2/5, R3/4, R1/6, and finally R7 (Meinertzhagen and Hanson, 1993). The R8 R-cells are the first to send axons through the optic stalk, followed by R1-R6 and finally R7. R1-R6 and R7 axons fasciculate with R8 until the first of two termination targets in the optic lobe, the lamina layer is reached. The R1-R6 axons terminate within the lamina layer, while the axons of both R8 and R7 pass through the lamina and terminate in temporary layers (Ting *et al.*, 2005) before proceeding to finally terminate in the medulla. Glial cells are essential for proper termination of R1-R6 axons in the laminal layer. Poeck *et al.* (2001) assessed whether the presence glial cells or neurons in the lamina were required by the R1-R6 axons for lamina termination using LOF mutations affecting either the laminal glial cells, or laminal neurons. Removal of glial cells from the lamina using mutants of the gene *non-stop*, that encodes a ubiquitin-specific protease controlling glial cell migration, resulted in a disruption of R1-R6 axonal targeting, while *hedgehog* mutants, that lack lamina neurons, have normal R1-R6 lamina termination patterns (Poeck *et al.*, 2001).

1.1.9 Use of the visual system as a model to examine axonal guidance and targeting

The *Drosophila* visual system is an excellent tool that can be used to identify molecules involved in axonal guidance and targeting. As R-cell axons terminate in a

limited number of layers in the optic lobe, the visual system provides a simple structure for the study of layer-specificity. The combination of anatomical simplicity, paired with powerful fly genetics, has resulted in the identification of a number of molecules necessary for proper R-cell guidance and targeting including: the two nuclear factors Brakeless and Runt; the cell-surface proteins Flamingo, PTP69D, the Insulin receptor, N-cadherin, and LAR; and the intracellular signal-transduction molecules Dock, Misshapen, Bifocal, Pak, and Trio. A detailed description of the identification and functional analysis of these proteins is provided in the next section (1.2) of this introduction.

In this thesis I have taken advantage of the *Drosophila* visual system as a model for the study of axonal guidance and targeting. In this introduction, I will describe the rational involved in my studies (section 1.4) following my review of literature describing known axonal guidance and targeting molecules in the visual system (section 1.2), and proteins known to be necessary for regulating axonal fasciculation in *Drosophila* (section 1.3).

1.2 Specific molecules involved in axonal guidance and target selection in the *Drosophila* visual system.

Previous studies have revealed roles for a number of nuclear factors, cell-surface proteins, and intracellular signal-transduction molecules in the guidance and targeting of R-cell axons in the *Drosophila* visual system. In this section I will describe the identification, mutant phenotypes, and functional models for each of these molecules.

1.2.1 Nuclear factors

1.2.1.1 Brakeless

Two groups independently identified *brakeless* (*bks*), a nuclear-protein encoding gene, in genetic screens for mutations that disrupted R-cell targeting in the *Drosophila*

visual system (Rao *et al.*, 2000; Senti *et al.*, 2000). Rao *et al.* (2000) conducted a screen of lethal P element insertions on the second chromosome while Dickson and colleagues (Newsome *et al.*, 2000a) performed a screen of mosaic flies where ethanemethanesulfonate (EMS)-induced mutations were rendered homozygous in the eye using the FLIP recombinase/FLP recombinase target (FLP/FRT) system. A third group identified *bks* in a behavioral screen for larval foraging behavior and named the *bks* gene *scribbler* (*sbb*) as they found *sbb* mutants demonstrated high amounts of turning behavior on agar plates in the absence of food (Yang *et al.*, 2000).

The Bks nuclear protein contains two nuclear localization signals beginning at amino acid 840 (PPAKRVK) and amino acid 841 (PAKRVKH) (Yang *et al.*, 2000) and was detected in the nuclei of R-cells in the developing eye-disc using immunohistochemistry (Rao *et al.*, 2000). The *bks* gene encodes two protein isoforms formed by alternative splicing. The shortest Bks isoform is BksA, a protein of 929 amino acids in length. The longer 2302 amino acid BksB isoform encompasses BksA and has an extended carboxyl (C)-terminus (Senti *et al.*, 2000). A single putative zinc finger located solely in the extended C-terminal portion of BksB indicates that BksB may have functions that differ from those of BksA. In a screen for modifiers of the *Merlin* epithelial cell proliferation phenotype, LaJeunesse *et al.* (2000) identified two *bks* alleles containing nonsense mutations in the extended BksB C-terminus that do not affect R-cell axon targeting. These results suggest that the BksA isoform function in the axonal targeting of R-cells, while the larger BksB functions in the regulation of epithelial cell proliferation.

In *bks* mutant larvae, most R1-R6 axons fail to terminate in the lamina layer and continue to grow into the medulla of the optic lobe. The R1-R6 loss-of-*bks* phenotype observed in third instar larva was found to persist until adulthood (Rao *et al.*, 2000; Senti *et al.*, 2000). However, *bks* does not affect R7 targeting to the medulla (Newsome, 2000). Expression of either BksA or BksB using an eye-specific driver GMR (Brand and Perrimon, 1993; Hay *et al.*, 1994; Rorth *et al.*, 1998) rescued the *bks* R1-R6 mutant phenotype, indicating a requirement for Bks in the eye.

A number of R-cell-specific markers were used to assess the possibility that *bks* alters the differentiation of R-cells. Antibodies against specific R-cell markers were used to compare marker expression in *bks* mutants to *wild-type* (*wt*) individuals (Rao *et al.*, 2000). That the expression of developmental markers examined in R7 (Kauffmann *et al.*, 1996), R8 (Reinke *et al.*, 1988), and R1 and R6 (Hayashi *et al.*, 1998) was indistinguishable from that of *wt* indicates that *bks* is not involved in R-cell differentiation.

The nuclear localization of Bks suggests that Bks is involved in a gene expression pathway that controls the expression of specific targeting determinants, for example, receptors for targeting signals or intermediate molecules involved in signal transduction pathways. That overexpression of Bks alone was unable to retarget R7 growth cones to the lamina (Senti *et al.*, 2000) suggests that Bks needs to cooperate with other nuclear factors in order to properly target axons to the lamina. Thus far, only one other nuclear protein has been found to interact with Bks—the transcription factor Runt (Run) (Kaminker *et al.*, 2002).

1.2.1.2 Runt

Run is a Runt domain family member (Kania *et al.*, 1990; Canon and Banerjee, 2000) transcription factor that is specifically expressed in R7 and R8 R-cells (Kaminker *et al.*, 2002). Kaminker *et al.* (2002) found in *bks* mutant eye discs that *run* was aberrantly expressed in R2 and R5 axons, in addition to the normal R7 and R8 expression found in *wt*. This observation led Kaminker *et al.* (2002) to propose that Bks represses the expression of Run in R2 and R5 R-cells. Consistent with this proposal, only the expression of Run in R2, R5, R7, and R8 axons (Tissot *et al.*, 1997) but not in R8 alone, in R1, R6, and R7, or in R3 and R4 led to the mistargeting of R-cells past the lamina into the medulla in third instar larva. Immunostaining of a variety of developmental protein markers indicated that Run misexpression in R2 and R5 did not result in the abnormal differentiation of R-cells (Reinke *et al.*, 1988; Kimmel *et al.*, 1990; Kauffmann *et al.*, 1996; Hayashi *et al.*, 1998). Together these results indicate that the severe R1-R6 mistargeting phenotype observed in *bks* mutants is due to a lack of suppression of *run* expression in R2 and R5 R-cells. The genes under transcriptional control of *bks/run* that are required for proper R1-R6 targeting remain unknown. Interestingly, the R1-R6 LOF phenotypes of *bks* and *run* are much more severe than those phenotypes previously described for molecules necessary for the proper targeting of R1-R6 axons to the lamina layer, including the receptor PTP69D (Garrity *et al.*, 1999) and the signal transduction molecules Dock (Garrity *et al.*, 1996), Misshapen (Ruan *et al.*, 1999), and Bifocal (Ruan *et al.*, 2002). This observation suggests that a number of genes encoding the necessary molecules for proper R1-R6 lamina-specific targeting are under the transcriptional control of *bks* and *run*, potentially including some proteins that are yet to be discovered.

1.2.2 Cell-surface proteins

1.2.2.1 Flamingo

Two groups independently described a role for *flamingo* (*fmi*), a cadherin-related cell surface protein (Usui *et al.*, 1999) in *Drosophila* visual system axonal targeting using different genetic screens (Lee *et al.*, 2003; Senti *et al.*, 2003). Zipursky and colleagues identified two *fmi* alleles in a genetic screen of mosaic flies with homozygous mutations found only in the eye (Lee *et al.*, 2001). The first step of this screen involved subjecting mosaic flies to an optomotor assay, a test that can assess the function of R1-R6 R-cells where flies must recognize a moving bar of visible light. Mosaic flies that failed to recognize the moving light—therefore those with a functional defect in R1-R6—were next subjected to a second test, the ultraviolet/visual light (UV/vis) choice assay in order to examine R7 function. In the UV/vis choice test, mosaic flies that failed to navigate toward a UV light source in a T-maze must have a functional R7 defect. Senti *et al.* (2003) identified nine alleles of *fmi* in a genetic screen of mosaic animals for mutations that affected R-cell targeting assessed by immunostaining the R-cell axons of third instar larval eye-brain complexes (Newsome *et al.*, 2000a).

While mutant *fmi* individuals displayed a defect in the optomotor response, a behavior that requires functional R1-R6 axons, normal R1-R6 target layer specificity was observed in both third-instar larval and adult stages (Lee *et al.*, 2003). However, DiI labeling of R-cell axon projections from single ommatidia in the developing pupa revealed that R1-R6 axons failed to form proper connections with their normal target neurons in the lamina (Lee *et al.*, 2003), indicating that the cause of the observed behavioral defect was likely due to the formation of improper connections by R1-R6

axons with lamina neurons, rather than a mistargeting of R1-R6 axons to the lamina. Electron microscopy of *fmi* mutant laminas demonstrated that normal synaptic structures formed between R-cell axons and lamina neurons despite the fact that the R-cell axons formed connections with incorrect partners (Lee *et al.*, 2003), suggesting that Fmi is required for the proper identification of synaptic partner by R1-R6 axons rather than being involved in synapse formation.

At the third instar larval stage, abnormally thick bundles of *fmi* mutant R7 and R8 axons were observed to project into the medulla. That the expression of Fmi in R8 axons, but not in R7 axons, was able to rescue this phenotype (Lee *et al.*, 2003) indicates that the R7 phenotype is non-cell autonomous. Lee *et al.* (2003) examined the requirement for Fmi in R7 axons by generating flies with single mutant R7 axons in an otherwise heterozygous or *wt* background using a technique called mosaic analysis with a repressible cell marker (MARCM) (Lee and Luo, 1999). MARCM analysis revealed that *fmi* mutant R7 axons target normally to the M6 medulla layer (Lee *et al.*, 2003). As Fmi has been demonstrated to have a role in homotypic cell adhesion (Usui *et al.*, 1999) and is expressed in R-cells (Lee *et al.*, 2003), Fmi may mediate interactions between R-cell axons to ensure proper target selection or between R-cell axons and target cells to promote the formation of stable connections. Thus far, Fmi is the sole protein known to be required for proper targeting of R8 axons, while PTP69D (Garrity *et al.*, 1999; Newsome *et al.*, 2000a), LAR (Clandinin *et al.*, 2001; Maurel-Zaffran *et al.*, 2001), and N-Cadherin (Lee *et al.*, 2001; Ting *et al.*, 2005) have all been shown to be necessary for the proper targeting of R7 axons.

1.2.2.2 PTP69D

Garrity *et al.* (1999) elected to investigate a potential role for the receptor tyrosine phosphatase PTP69D in the guidance and targeting of R-cell axons as previous studies (Garriety *et al.*, 1999) had indicated an involvement of tyrosine phosphorylation signaling in the guidance and targeting of R-cells. PTP69D has a large extracellular domain that contains two immunoglobulin (Ig) domains and three fibronectin III (FNIII) type domains while the intracellular domain of PTP69D contains two protein tyrosine phosphatase (PTP) catalytic domains.

Immunostaining of *Ptp69D* larval mutants revealed a discontinuous lamina layer and abnormal axonal bundles entering the medulla. By specifically labeling the R2-R5 axons of third instar larva, Garrity *et al.* (1999) estimate that 20-25% of *Ptp69D* mutant R2-R5 axons mistarget into the medulla. X-ray induced mitotic recombination was used to generate mutant *Ptp69D* patches of retinal tissue. Abnormal extension of R1-R6 axons into the adult medulla was observed in mosaic animals, indicating a requirement for PTP69D in R-cell axons and not the target region. Garrity *et al.* (1999) performed rescue experiments by expressing either full-length *wt* or mutant forms of *Ptp69D* in *Ptp69D* mutants. The results of rescue experiments were quantified by estimating the percentage of ommatidia that extend R2-R5 axons into the medulla, visualized in third instar larva using a R2-R5 marker. While expression of *wt*, PTP1-inactive, or immunoglobulin domain-lacking forms of PTP69D were all found to rescue the *Ptp69D* phenotype, mutant forms of PTP69D either completely lacking phosphatase activity or missing the FNIII domains failed to rescue. These results indicate a requirement for the FNIII

domains, possibly to bind ligands in the lamina, in order to stimulate PTP69D phosphatase activity.

A clear model of PTP69D function cannot be interpreted solely from the results of Garrity *et al.* (1999). Garrity *et al.* (1999) found that in the absence of PTP69D, or in the presence of mutant-PTP69D lacking phosphatase activity, R1-R6 axons fail to correctly stop at the lamina layer. One possible explanation for this result is that PTP69D, expressed by R1-R6 growth cones, may instruct R1-R6 axons to stop correctly upon stimulation by an unknown ligand located in the lamina. According to this hypothesis, the overexpression of PTP69D in R7 and R8 axons may lead to the aberrant early termination of R7 and R8 axons in the lamina layer. However, Garrity *et al.* (1999) found that PTP69D overexpression in R7 and R8 did not cause a change in R7 and R8 phenotype. One possible explanation for this result is that PTP69D and the intracellular molecules needed for PTP69D function may not normally be expressed in R7 and R8 growth cones. A second possible explanation for the results of Garriety *et al.* (1999) is that PTP69D may act in a non-specific rather than an instructive manner. According to a model for non-specific function, PTP69D signaling would permit follower R-cell axons to separate from the R8 pioneer axon and enable the follower axons to respond to more specific signals. The overexpression of a permissive-acting receptor in R7 and R8 axons would not cause the retargeting of R7 and R8 axons to the medulla. To distinguish between these two possibilities, the *wt* expression and function of PTP69D in R7 axons must be assessed.

Newsome *et al.* (2000) labeled *Ptp69D* mutant R7 axons and observed the early termination of R7 axons at the R8 M3 medulla layer. This result is consistent with a

permissive model for PTP69D function as the removal of PTP69D may cause R7 axons to remain tightly fasciculated with R8 pioneer axons. No longer able to reach the normal R7 terminal region in the M6 medulla layer, *Ptp69D* mutant R7 axons consequently terminate abnormally with R8 in the M3 medulla layer. Two further experiments are required to support the idea that PTP69D functions in a permissive manner in R-cell axons. First, PTP69D expression must be assessed specifically in R7 axons. If PTP69D is not normally expressed in R7 axons, then the early R7 termination phenotype observed by Newsome *et al.* (2000) cannot be the result of PTP69D function in R7 axons. Second, single-mutant PTP69D R7 axons must be generated to test for a cell-autonomous function of PTP69D using the MARCM technique. Lacking R7-specific phenotypic analysis, the autonomy PTP69D function in R7 axons is unknown. Without further analysis of PTP69D expression and autonomy, one cannot firmly conclude that PTP69D acts in a permissive manner in R-cell axons.

1.2.2.3 LAR

A role for the receptor tyrosine phosphatase LAR (Streuli *et al.*, 1989) in visual system development was independently identified in three genetic screens (Newsome *et al.*, 2000a; Clandinin *et al.*, 2001; Maurel-Zaffran *et al.*, 2001). Zipursky and colleagues isolated alleles of *LAR* in the same behavioral genetic screen used to isolate alleles of *N-cadherin* (Lee *et al.*, 2001) and *fmi* (Lee *et al.*, 2003). Dickson and colleagues identified four alleles of *LAR* in the screen of mosaic third instar larva that isolated alleles of *bks*, *ptp69D*, and *trio* (Newsome *et al.*, 2000a). Maurel-Zaffran *et al.* (2001) found two *LAR* alleles in a screen of adults' mosaic for EMS-induced mutations that were analyzed by the examination of sectioned heads with stained R-cell axon projections.

LAR expression was detected in all R-cells, as well as in other cell types in the optic lobe at pupal and adult developmental stages (Clandinin *et al.*, 2001; Maurel-Zaffran *et al.*, 2001). While the R1-R6 axonal projection pattern in *LAR* mosaic third-instar larva was found to be normal, DiI labeling of single ommatidia in pupa revealed a failure of R1-R6 axons to form proper connections with the correct target cells (Clandinin *et al.*, 2001). MARCM analysis was used to visualize single *LAR* mutant R1-R6 fibers. Fifty percent of *LAR* mutant axons failed to extend outward toward the appropriate target-cells (Clandinin *et al.*, 2001), indicating a cell-autonomous requirement for LAR in R1-R6 axons.

The LAR phenotype was assessed in pupal stages following 15 and 35 hrs of development (Clandinin *et al.*, 2001). Following 15 hrs of pupal development, Clandinin *et al.* (2001) observed an extension of R7 axons beyond the R8 terminal region. R7 terminals were found to terminate at the R8 layer, or in between the R7 and R8 layers only in older posterior regions of the medulla (Clandinin *et al.*, 2001). The percentage of collapsed or absent R7 axons following 35 hrs of pupal development in younger regions of the medulla was quantified and compared to older medulla regions. Clandinin *et al.* (2001) found a positive co-relation between R7 axon age and collapse or absence. This result indicates that R7 axons initially extend toward the normal R7 terminal region of the medulla, and subsequently retract from this layer during pupal development. The examination of *LAR* mosaic adult axonal projection patterns revealed the aberrant termination of R7 axons in the superficial M3 layer of the medulla, while R1-R6 and R8 axons were found to terminate in the correct layers (Maurel-Zaffran *et al.*, 2001).

The similarity of the *LAR* and *Ptp69D* mutant R7 R-cell phenotypes suggests that both molecules may act in similar biochemical pathways (Newsome *et al.*, 2000a; Maurel-Zaffran *et al.*, 2001). In order to test whether *LAR* and *PTP69D* act in independent or in similar signal transduction pathways, Maurel-Zaffran *et al.* (2001) expressed either PTP69D or LAR using an eye-specific promoter in *LAR* and *Ptp69D* mutants. Maurel-Zaffran *et al.* (2001) found while LAR expression could rescue either *LAR* or *Ptp69D* mutants, PTP69D expression could only rescue the *Ptp69D* mutant phenotype. This result indicates that LAR and PTP69D may interact with a common set of signaling partners, though some signaling partners may be unique to LAR. To test whether the signaling partners unique to LAR are upstream, extracellular ligands or downstream, intracellular molecules, chimeric receptors were created in which the LAR/PTP69D cytoplasmic domains had been exchanged and tested for the ability to rescue the *LAR* mutant phenotype (Maurel-Zaffran *et al.*, 2001). Only the chimeric receptor consisting of the LAR extracellular domain and the PTP69D intracellular domain was found to rescue *LAR*, indicating that LAR interacts with specific extracellular cues, though both LAR and PTP69D share a common intracellular pathway.

While the ligand(s) unique to LAR are unknown, Maurel-Zaffran *et al.* (2001) provide evidence that LAR may signal through the intracellular molecules Trio or Enabled (Ena). By reducing the dosage of either the Rho family guanine nucleotide exchange factor Trio, or the substrate of the Abl cytoplasmic tyrosine kinase Ena by 50% in a sensitive LAR hypomorphic background, Maurel-Zaffran *et al.* (2001) observed the enhancement of the *LAR* phenotype. Maurel-Zaffran *et al.* (2001) found that expression of Ena or Trio in a *LAR* hypomorphic background led to the suppression of the *LAR*

phenotype. These results are consistent with a model in which LAR may signal intracellularly through Ena and/or Trio in R7 axons in order to stabilize the proper connection of R7 with target cells. While PTP69D may initially target R7 axons to the M6 medulla layer, a lack of LAR in R7 axons leads to the instability of R7 terminals leading to R7 axonal retraction, possibly through Ena or Trio. The observation that R1-R6 axons correctly reach the lamina layer but fail to form proper connections with lamina neuron target cells also supports the idea that LAR promotes the stability of axons with target cells. In order to test this hypothesis, the identification of the LAR ligand and removal of this ligand from R1-R6 lamina neuron target cells will be necessary. If LAR interacts with a ligand localized to R1-R6 axon target cells, the removal of the ligand from lamina neurons will result in a LOF phenotype similar to that of *LAR* mutants.

1.2.2.4 N-Cadherin

Zipursky and colleagues identified a role for N-Cadherin in the visual system by means of a two-step behavioral genetic screen using mosaic flies with eye-specific mutations (Lee *et al.*, 2001). Both *fmi* (Lee *et al.*, 2003) and *LAR* (Clandinin *et al.*, 2003) were also identified using this behavioral screen (Lee *et al.*, 2001).

Immunohistochemistry revealed that N-Cadherin is expressed on all R1-R8 axons during the third-instar larva stage and throughout pupal development (Lee *et al.*, 2001). In *N-cadherin* mutants, a disrupted array of R7 and R8 axons in the medulla was observed along with an abnormal spacing and lack of normally expanded R4 axon terminals in the lamina, though R2-R5 axons still terminated within the lamina. The behavioral defects in *N-cadherin* mutants observed using the optomotor assay appears to be caused by a failure of R1-R6 axons to extend out from their ommatidial bundle to

innervate normal lamina targets—a phenotype observed by the DiI labeling of single ommatidia of *N-cadherin* mosaic pupa (Lee *et al.*, 2001). Prakash *et al.* (2005) used the MARCM technique to analyze the projection of individual *N-cadherin* mutant R1-R6 axons and observed abnormal axonal extension toward targets within the lamina. This evidence supports the idea that *N-cadherin* is required autonomously in all R1-R6 cells.

Prakash *et al.* (2005) have also demonstrated a requirement for N-Cadherin in the target cells of R-cell axons. The MARCM method was used to create mosaics that lacked N-Cadherin expression in L1-L5 lamina neurons, the target cells of R1-R6 axons (Prakash *et al.*, 2005). While the *wt* R-cell axons correctly reached the *N-cadherin* mutant lamina neurons, the R-cell axons abnormally connected to the lamina neurons. This result demonstrates a requirement for N-Cadherin in lamina neurons for proper R-cell targeting. However, N-Cadherin is not required for individual R-cell axons to meet specific lamina neuron partners (Prakash *et al.*, 2005). This surprising finding indicates that N-Cadherin functions generally to attract R1-R6 axons toward final lamina neuron target cells, but does not act to instruct single R-cell axons to target individual lamina targets. The results of Prakash *et al.* (2005) support a permissive model where N-Cadherin is required to allow R-cell axons to reach lamina neuron targets, while more specific unknown signals are required to instruct R-cell axons to target specific lamina neurons. The identification of targeting molecules that specifically instruct R-cell axons to establish point-to-point connection to lamina neuron target cells will shed more light on the molecular mechanism of R1-R6 axon targeting.

Lee *et al.* (2001) examined the *N-cadherin* mutant phenotype of R7 and R8 axons. At adulthood, mutant R7 and R8 axons fail to form a normal array, often terminating at

the outer edge of the medulla (Lee *et al.*, 2001). The MARCM method was used to analyze single *N-cadherin* mutant R7 axons. In adults, single *N-cadherin* mutant R7 axons were consistently observed to aberrantly terminate in the R8 recipient layer, M3, rather than the normal M6 layer of the medulla (Lee *et al.*, 2001). The function of R7 mutant axons was analyzed by first selectively killing non-mutant R7 cells with a transgene, *PANR7-Tox* that expressed the tetanus toxin light chain in R7 cells. Flies with mutant R7 cells were next subjected to the UV/vis choice test, and were found to no longer be able to recognize UV light, a behavior that is dependant upon proper R7 function (Lee *et al.*, 2001). The lack of *N-cadherin* mutant R7 axons in the M6 layer of the adult medulla could either be due to the retraction of instable R7 axons from the correct layer during pupation, or reflect an inability of mutant R7 axons to reach the final M6 target layer. To distinguish among these possibilities, Ting *et al.* (2005) used the MARCM technique to analyze single *N-cadherin* mutant R7 axons in pupa from 17% to 35% of development. Ting *et al.* (2005) found that 55% of mutant R7 axons failed to reach a temporary layer that normal R7 axons reach before extending to final M6 medulla positions. This result is in marked contrast to pupal mutants for the receptor tyrosine phosphatase *LAR*, discussed in the preceding section, in which R7 axons terminate correctly at the R7-temporary medulla layer at both 17% and 35% of pupal development (Ting *et al.*, 2005).

The results of Ting *et al.* (2005) indicate that *N-cadherin* but not *LAR* is necessary for proper targeting to the R7 temporary medulla layer, and that N-Cadherin is required by R7 axons to reach the M6 medulla layer. Whether N-Cadherin is also required in medulla target neurons for R7 target selection, however, remains unknown. Future work

will be necessary to determine if N-Cadherin promotes the general attraction between R7 axons and their target neurons in a way similar to its action in R1-R6 target selection. For instance, the expression of N-Cadherin by cells in the temporary R7 terminal region would permit R7 axons to reach the correct R7 temporary terminal areas and then allow R7 axons to interpret specific instructions from unknown cues. Ultimately, the identification of specific signaling molecules that work together with N-Cadherin to specifically target R-cell axons will be necessary to fully understand N-Cadherin function.

1.2.2.5 DInR

Song *et al.* (2003) demonstrated a role for the insulin receptor (DInR) in visual system development when they isolated the adaptor molecule Dreadlocks (Dock), known to be involved in R-cell targeting, in a yeast two-hybrid (Y 2-H) library screen (Bartel and Fields, 1997) using the DInR intracellular domain as "bait." The role of Dock as an axonal targeting molecule will be discussed in the following section entitled "Intracellular signal-transduction molecules" (section 1.2.3.1).

Song *et al.* (2003) performed a number of biochemical tests to further characterize the interaction between DInR and Dock. Y 2-H binding assays were used to examine the domain requirement of Dock for DInR binding (Song *et al.*, 2003). Deletion of the Dock Src-homology 2 (SH2) domain, or the three SH3 domains led to a decreased Dock-DInR interaction by 50%, indicating the necessity for both SH2 and SH3 domains of Dock to strongly bind to DInR. Dock was unable to bind to a kinase-inactive form of DInR in a Y 2-H binding assay, indicating that Dock can only interact with DInR following an autophosphorylation event (Song *et al.*, 2003). The association of DInR and Dock in fly

lysate was demonstrated by the immunoprecipitation of DInR from the lysate of *wt* adult heads and third instar larval eye-brain complexes using an anti-human Nck antibody that also recognizes Dock. The inverse immunoprecipitation experiment using an anti-DInR antibody was also successfully performed. These biochemical results indicate that only activated DInR can bind to Dock and these proteins are associated in fly tissue extract. Next, Song *et al.* (2003) examined the expression pattern and mutant phenotype of *dinr* and tested the potential *in vivo* interaction of DInR and Dock.

Song *et al.* (2003) found abnormal gaps and densely packed regions in the lamina layer and a failure of growth cones to expand in the medulla of *dinr* mutant third instar larva. The loss-of-*dinr* phenotype is similar to that described for *dock* mutants (Garrity *et al.*, 1996), supporting the hypothesis that DInR and Dock may act in the same pathway to regulate R-cell axon guidance. In order to test for genetic interaction between *dinr* and *dock*, Song *et al.* (2003) examined the projection patterns of adults' transheterozygous for *dinr* and *dock*. While the adult projection patterns of individual heterozygotes for *dinr* or *dock* appear similar to *wt*, transheterozygotes for *dinr* and *dock* display an abnormal phenotype similar to that of *dinr*, featuring gaps in the R7 terminal layer and crossed fibers, supporting the idea that DInR and Dock function in the same biochemical pathway.

While Song *et al.* (2003) have provided biochemical and genetic evidence to suggest that DInR interacts with Dock and that DInR is required for proper R-cell guidance, the demonstration that a DInR ligand is expressed in the *Drosophila* visual system remains to be shown and is necessary to conclude that DInR normally acts as a guidance receptor. Expression of the known DInR ligand, insulin, involved in glucose

metabolism, is not restricted to the visual system (Brogiolo *et al.*, 2001). Were DInR to function as a guidance receptor, either insulin must be demonstrated to be expressed in an area where it may act as a guidance signal for the proper projection of R-cells, or alternatively, a novel DInR ligand must be identified. As the expression of a DInR ligand in the visual system has not yet been demonstrated, the possibility that the LOF phenotype exhibited by *dinr* mutants may be secondary to a defect in normal R-cell metabolism cannot be ruled out.

1.2.3 Intracellular signal-transduction molecules

1.2.3.1 Dock

Garrity *et al.* (1996) identified two alleles of the *dreadlocks* (*dock*) gene in a genetic screen that involved the examination of the R-cell projection patterns of 535 P-element lines at the third instar larval stage. Dock, an SH2 and SH3 domain-containing adaptor molecule was shown to be expressed in R-cell axons, the central neuropil, and weakly in medulla neurons. R-cell axons of *dock* mutants demonstrated both pathfinding and targeting defects. R-cells with pathfinding defects were observed that crossed over the paths of neighbouring axons and that were abnormally fasciculated leading to the formation of gaps in the lamina layer (Garrity *et al.*, 1996). R-cell growth cones were observed to clump in the lamina, while thick axonal bundles passed through the lamina to hyperinnervate the medulla. Mosaic flies homozygous for *dock* mutations only in the eye were generated by X-ray-induced mitotic recombination (Garrity *et al.*, 1996). In adult *dock* mosaics the abnormal crossing of mutant fibers resulted in gaps in the medulla array of R-cells. Labeling of R1-R6 axons in the adult revealed the aberrant extension of R1-R6 axons into the medulla—a failure of R1-R6 axons to terminate in the appropriate

target layer. The *dock* mutant phenotype was rescued by the expression of full-length Dock in the eye (Garriety *et al.*, 1996; Rao and Zipursky, 1998).

Analysis of the *dock* LOF phenotype indicates a requirement for Dock in R-cells for the maintenance of proper retinotopic organization and targeting of R-cells into the optic lobe. The fact that Dock contains SH2 and SH3 domains suggest a role for Dock to act downstream from a receptor tyrosine kinase (Pawson, 2004; Machida and Mayer, 2005). The putative receptor tyrosine kinase that would act upstream from Dock in R1-R6 axons could recognize a "stop" signal presented by lamina target cells, triggering a tyrosine phosphorylation event and thus recruitment of Dock to the plasma membrane. Thus, in *dock* mutants, R1-R6 axons fail to signal information from the receptor tyrosine kinase to the actin cytoskeleton within R1-R6 growth cones, resulting in a failure of R1-R6 axons to properly stop at the lamina. While the definitive upstream molecule that interacts with Dock is unknown, two molecules downstream of Dock that ultimately lead to the transduction of information to the growth cone cytoskeleton have been identified: the serine/threonine kinase misshapen (Msn) (Ruan *et al.*, 1999) and the p21-activated kinase (Pak) (Hing *et al.*, 1999). Both Msn and Pak will be discussed below.

1.2.3.2 Misshapen

The serine/threonine kinase Msn was selected as a candidate for study (Ruan *et al.*, 1999) as the Msn vertebrate homologue Nck-interacting kinase (NIK) was shown to bind to Nck, the vertebrate homologue of Dock in cultured cells (Su *et al.*, 1997). Also, the *Caenorhabditis elegans* homologue of Msn called Mig-15 was found to regulate changes in cell morphology (Treisman *et al.*, 1997; Su *et al.*, 1998), indicating the potential role for Msn to regulate growth cone morphology. Ruan *et al.* (1999) found that

Msn is expressed in R-cell axons and growth cones and observed a similar LOF phenotype in *msn* mutants and mosaics as had previously been described for *dock* mutants (Garrity *et al.*, 1996). Loss-of-*msn* resulted in an uneven lamina layer with gaps, in addition to the appearance of large bundles in the medulla. The labeling of R1-R6 axons revealed the aberrant growth of R1-R6 into the medulla (Ruan *et al.*, 1999). Additionally, Ruan *et al.* (1999) found a dosage-sensitive GOF phenotype when they overexpressed *msn* using an eye-specific promoter. Consistent with the failure of R1-R6 axons to properly terminate at the lamina in the absence of Msn, Msn over-expression induced the early termination of R-cell axons prior to reaching the lamina (Ruan *et al.*, 1999).

The similar expression patterns and LOF phenotypes of *dock* and *msn* led Ruan *et al.* (1999) to study the potential biochemical interaction of the two molecules. An immobilized glutathionine *S*-transferase (GST)-tagged fragment of Msn that contains multiple proline-rich PXXP motifs for SH3 domain binding was found to precipitate Dock from adult fly lysates. Additionally, an anti-Dock antibody was able to immunoprecipitate Msn from the lysate of third-instar eye-brain complexes and adult heads. Finally, a Y 2-H binding assay was used to determine the domain requirements for Dock-Msn physical interaction. Ruan *et al.* (1999) demonstrate that the proline-rich domain of Msn binds to the SH3-1 and SH3-2 domains of Dock, and weakly interacts with the Dock SH3-3 domain. These results led Ruan *et al.* (1999) to examine the potential *in vivo* genetic interaction of Msn and Dock.

The reduction of 50% of *dock* dosage in an *msn* hypomorphic phenotype resulted in an enhancement of the loss-of-*msn* phenotype (Ruan *et al.*, 1999), consistent with the

possibility that Dock and Msn function in the same pathway (Guarente, 1993). To further test this idea, Ruan *et al.* (1999) next overexpressed Msn in *dock* mutants, and observed a deterrence of R1-R6 axons from entering the medulla, a result that is also consistent with an activating role for Msn by Dock. However, Ruan *et al.* (1999) also observed early termination of R1-R6 axons when overexpressing Msn in *dock* mutants; this result suggested that Dock could also negatively regulate Msn. Consistent with a possible negative regulatory role for Dock, the co-overexpression of both Msn and Dock using a eye-specific promoter led to the suppression of the Msn GOF phenotype (Ruan *et al.*, 1999). This inhibition of Msn by Dock is dependant upon upstream signals, as the overexpression of a Dock mutant lacking the SH2 domain can no longer suppress the Msn GOF phenotype.

The genetic and biochemical evidence provided by Ruan *et al.* (1999) suggests that in R1-R6 axons Msn can potentially be activated by a Dock-mediated "stop" signal. However, the observation that Dock can also negatively regulate Msn increases the complexity of Dock/Msn interaction. Multiple upstream molecules may possibly signal through Dock to either activate or inhibit Msn function. Currently, the molecules that act upstream of Dock are unknown. Thus far, the only known Msn-interacting molecule involved in R-cell targeting is the cytoskeletal regulator Bifocal (Bif) (Ruan *et al.*, 2002)

1.2.3.3 Bifocal

Ruan *et al.* (2002) identified a role for *bif* in R-cell targeting when they tested a set of genes that previously had been shown to regulate cytoskeletal dynamics in *Drosophila* (Bahri *et al.*, 1997; Sisson *et al.*, 2000) for genetic interaction with *msn*. Reduction of 50% *bif* dosage in larva that overexpressed *msn* resulted in suppression of

the *msn* GOF phenotype, suggesting that Bif functions downstream of Msn in R-cell growth cones. Ruan *et al.* (2002) observed Bif expression in cultured R-cells and described *bif* LOF and GOF phenotypes similar to those shown for *dock* (Garrity *et al.*, 1996) and *msn* (Ruan *et al.*, 1999).

Ruan *et al.* (2002) performed biochemical analysis to distinguish whether the genetic interaction of Msn and Bif reflected a direct physical interaction or an indirect interaction involving one or more intermediate molecules. Direct physical interaction of Msn and Bif was observed when an immobilized Bif C-terminal fragment fused to GST was used to precipitate both purified Msn and Msn from COS-7 cells. As Msn is a serine/threonine kinase, an *in vitro* phosphorylation assay was performed to test the ability of Msn to phosphorylate Bif (Ruan *et al.*, 2002). Indeed, Msn was found to phosphorylate both C- and amino (N)-terminal Bif peptides. Kinase activity was found to be essential for Msn function in R-cell growth cones, as a kinase-defective *msn* mutant was unable to rescue the *msn* mutant phenotype.

To determine the action of Msn and Bif on the growth cone cytoskeleton, Ruan *et al.* (2002) expressed Bif and Msn in COS-7 cells. The expression of Bif alone in COS-7 cells dramatically increases the formation of filamentous actin (F-actin) and filopodia-like structures (Ruan *et al.*, 2002). Bif co-localizes with F-actin, raising the possibility that Bif directly binds to F-actin. In cells co-expressing Msn and Bif, although the amount of F-actin is still significantly higher than control cells, the structure of F-actin was altered. Instead of forming long, fine fibers, F-actin was organized into large aggregates. The filopodia-like structures also became much shorter. In order to examine whether the effect of Msn on filopodia formation and shape required kinase-activity,

COS-7 cells were also co-transfected with Bif and kinase-defective Msn (Ruan *et al.*, 2002). Cells co-transfected with kinase-defective Msn and Bif appeared similar to those transfected with Bif alone. These results indicate that Bif can promote actin polymerization resulting in filopodia formation, while Msn can modulate Bif activity in a kinase activity-dependant manner.

The similar LOF phenotypes of *dock* (Garrity *et al.*, 1996), *msn* (Ruan *et al.*, 1999), and *bif* (Ruan *et al.*, 2002) support the idea that all three molecules interact in a biochemical pathway necessary for the proper termination of R1-R6 axons at the lamina layer. That transfection of COS-7 cells with Bif promotes filopodia formation, while co-transfection of COS-7 cells with both Bif and Msn modulate the Bif phenotype, is consistent with a model in which activated Msn could diminish the ability of Bif to promote filopodia formation in the growth cone, thus allowing R1-R6 axons properly terminate at the lamina layer. This model would first involve the recognition of a "stop" signal by an unknown receptor at the plasma membrane, triggering a tyrosine phosphorylation event. Dock could be recruited to the plasma membrane to bind to phosphotyrosine via the SH2 domain (Machida and Mayer, 2005). Next Msn would directly interact with activated Dock, resulting in Msn activation, finally triggering Bif phosphorylation by Msn ultimately resulting in proper termination of R1-R6 axons at the lamina.

1.2.3.4 Pak

Hing *et al.* (1999) decided to study the kinase Pak as a candidate molecule downstream of Dock in R-cell growth cones because the mammalian Pak homologue had previously been shown to interact with Nck, the Dock mammalian homologue (McCarty,

1998). Pak kinase is composed of an N-terminal regulatory region and a C-terminal kinase domain (Frost *et al.*, 1998; Zhao *et al.*, 1998). The N-terminal regulatory region consists of an N-terminal PXXP sequence, a Cdc42/Rac interactive binding (CRIB) motif, and a proline-rich motif that in mammals has been shown to be bound to the guanine nucleotide exchange factor Pix (Manser *et al.*, 1998).

Hing *et al.* (1999) found that both Dock and Pak are expressed in R-cell axons and growth cones, in addition to being expressed in neuronal cell bodies in the lamina and medulla cortical region. The LOF phenotypes of *Pak* and *dock* mutants are similar, featuring the uneven spreading of axonal fibers within the lamina and medulla and the termination of thick, blunt fascicles in the medulla (Hing *et al.*, 1999), consistent with a role for Dock and Pak interaction in the same pathway. The physical interaction of Pak and Dock was demonstrated using a Y 2-H binding assay and by the immunoprecipitation of Pak from S2 cell lysate using an anti-Dock antibody (Hing *et al.*, 1999).

By transgene rescue experiments and the examination of *pak* missence mutations, Hing *et al.* (1999) determined the domains necessary for Pak function in R-cells. An examination of flies that have a *Pak* missence mutation in the Dock-binding site revealed a similar phenotype to that of strong *Pak* mutants (Hing *et al.*, 1999). Hing *et al.* (1999) also found the expression of Pak transgenes mutant for either kinase ability or the capacity to bind to Rac/Cdc42 were unable to rescue the *pak* mutant phenotype, while expression of *wt* Pak did lead to rescue. These results indicated that the binding of Dock and Rac/Cdc42 to Pak, and a need for kinase ability are necessary for Pak function.

Hing *et al.* (1999) generated a dominant GOF form of Pak by tethering the molecule to the plasma membrane using the Src1 myristylation signal. The Pak GOF

phenotype is dosage sensitive, as the expression of four copies of myristylated Pak resulted in the severe disruption of the R-cell axonal projection pattern, including the migration of some R-cell bodies into the medulla (Hing *et al.*, 1999). Myristylated-Pak was shown to have *wt* Pak activity, as the expression of a single copy using an eye-specific promoter was sufficient rescue the *pak* mutant phenotype (Hing *et al.*, 1999). Together, these results demonstrated that Pak must be localized to the plasma membrane for R-cell function. Hing *et al.* (1999) next tested whether the function of Dock is to recruit Pak to the plasma membrane. Hing *et al.* (1999) confirmed this hypothesis by largely rescuing the *dock* mutant phenotype when expressing myristylated-Pak in a *dock* mutant background.

The biochemical and genetic results of Hing *et al.* (1999) revealed that Dock functions to recruit Pak to the plasma membrane, where Pak may participate in further downstream signaling events. Dock activation by an unknown receptor tyrosine kinase may recruit Dock to the plasma membrane, activate Dock, and trigger the binding of Dock to Pak. As Pak signaling is dependent upon both Pak kinase activity and the binding of Pak to Cdc42/Rac (Hing *et al.*, 1999), and guanine nucleotide exchange factors (GEF) are necessary for Cdc42/Rac activation (Mueller, 1999), Pak must be bound to a GEF that can promote the exchange of GDP for GTP of Pak-bound Cdc42/Rac, thus activating Cdc42/Rac. In turn, GTP-bound Cdc42/Rac would activate Pak leading to the phosphorylation of an unknown downstream ligand, ultimately resulting in the regulation of the actin cytoskeleton in R-cell growth cones. The GEF that acts in concert with Pak to direct R-cell axon guidance has since been demonstrated to be Trio (Newsome *et al.*, 2000b).

1.2.3.5 Trio

Dickson and colleagues identified nine alleles of *trio* in their saturation mutagenesis screen to identify genes that affect R-cell axon guidance (Newsome *et al.*, 2000a). *Drosophila* Trio is comprised of "two Dbl homology (DH) domains, each with a flanking pleckstrin homology (PH) domain" and these tandem DH-PH domains are referred to as GEF1 and GEF2 (Newsome *et al.*, 2000b). Newsome *et al.* (2000b) used an anti-Trio antibody to show that Trio is found at or near the plasma membrane of multiple cell types in third-instar eye imaginal discs. Dissociated R-cells were cultured *in vitro* and stained with an anti-Trio antibody to reveal Trio expression by R-cell growth cones because endogenous Trio expression in the optic lobe made the examination of R-cell axons difficult (Newsome *et al.*, 2000b).

Newsome *et al.* (2000b) found that loss-of-*trio* led to a similar phenotype to that of *dock* (Garrity, 1996) and *Pak* (Hing, 1999) indicating that Trio, Dock, and Pak may act together in a biochemical pathway. In the third instar larval stage, *trio* and *Pak* mosaics exhibited disorganized projection patterns including gaps in the lamina layer and the formation of bundles in the medulla (Newsome *et al.*, 2000b). Newsome *et al.* (2000b) observed axons bypass the medulla and terminate in between the medulla and lobula of adult *trio* and *Pak* mosaic optic lobes. Use of a R7 specific marker also revealed the aberrant misrouting of R7 axons around the medulla (Newsome *et al.*, 2000b). These results indicate the necessity of Trio and Pak in proper R-cell guidance.

A rescue experiment using mutant forms of Trio was performed to identify the GEF domain necessary for R-cell function. Newsome *et al.* (2000b) found that expression of mutant Trio with a non-functioning GEF1 GTPase binding site was unable

to rescue *trio* mutants, while a similar mutant Trio with a non-functional GEF2 site could rescue the loss-of-*trio* phenotype. This result indicates that the Trio GEF1 site is essential for R-cell function.

GEFs are a class of molecule necessary for the activation of Rho family GTPases by promoting the exchange of GTP for bound GDP (Mueller, 1999). As Trio function in R-cells is dependent upon a GEF1 domain, Newsome *et al.* (2000b) next sought to identify the particular Rho family GTPases activated by Trio. Newsome *et al.* (2000b) completed guanine nucleotide release assays to determine which Rho GTPase of Cdc42, RhoA, RhoL, Rac1, Rac2, and the Mig-two-like GTPase Mtl would release bound ^{3}H-GDP following a 15 minute incubation with a Trio GEF1-GST fusion protein. GEF1 stimulated GDP release from Rac1, Rac2, and Mtl suggesting that Rac1, Rac2 and Mtl may be the Rho family GTPases that act in Pak/Trio signaling. Next, Newsome *et al.* (2000b) tested the abilities of GTP bound immobilized GST-Rac1, GST-Rac2, or GST-Mtl to pull-down Pak from COS-7 cell lysate. Newsome *et al.* (2000b) found that only Rac1 and Rac2 but not Mtl could pull-down Pak. Similarly, Newsome *et al.* (2000b) found that only constitutively active Rac1 but not Mtl could interact with Pak in a Y 2-H binding assay. Together these results suggest that only Rac1 and Rac2 can directly activate Pak.

Newsome *et al.* (2000b) overexpressed the Trio GEF1 domain in the eye and observed a dominant GOF phenotype consisting of axonal bundling in the optic stalk and a disorganized projection pattern within the optic lobe. The potential *in vivo* genetic interaction of *trio* and each of the Rho family GTPases Rac1, Mtl, Cdc42, and RhoA was assessed by expression of the GTPases with an eye-specific promoter in the Trio GEF1

GOF background (Newsome *et al.*, 2000b). Both Rac1 and Mtl *GMR*-expression in the GEF1 GOF background led to enhancement of the GEF1 phenotype, indicating genetic interaction of Rac1 and Mtl with Trio. Finally, Newsome *et al.* (2000b) examined the dosage-sensitive interaction of *trio*, *dock*, and *Pak*. The removal of 50% of *trio* in a hypomorphic *dock* background resulted in the enhancement of the loss-of-*dock* phenotype—Newsome *et al.* (2000b) found an increase in the occurrence from the medulla bypass phenotype from 14% in *dock* hypomorphs to 87%. Newsome *et al.* (2000b) also observed genetic interaction between *trio* and *Pak*. The reduction of 50% *Pak* dosage in a *trio* hypomorphic background led to an increase in the medulla bypass phenotype from 7% in *trio* hypomorphs to 34-42% when *Pak* dosage was reduced. These results further support the idea that together, Dock, Pak, and Trio interact in the same biochemical pathway.

A two-signal model can account for the interaction of Dock, Pak, and Trio in the guidance of a R-cell growth cone (Newsome *et al.*, 2000b). According to this model, a first unknown signal would activate Trio that in turn can activate Rac1 or 2, the only Rho GTPases shown to interact with Pak both *in vitro* and *in vivo* (Newsome *et al.*, 2000b). A second signal would allow Dock to recruit Pak to the plasma membrane. In areas of the growth cone that are activated by both signals, activated Rac could bind to Pak, thus stimulating Pak kinase activity. Ultimately, the cytoskeleton could be regulated in such a manner as to steer the growth cone toward an appropriate direction.

The Dock-Msn-Bif and Dock-Pak-Rac-Trio signaling pathways perform essential but distinct functions in R-cell growth cones. The mistargeting of R1-R6 axons into the medulla was observed in *dock* (Garrity *et al.*, 1996), *msn* (Ruan *et al.*, 1999), and *bif*

(Ruan *et al.*, 2002) mutants. This result suggests a requirement for the Dock-Msn-Bif pathway in the lamina-specific targeting of R1-R6 axons. By contrast, in both *Pak* (Hing *et al.*, 1999) and *trio* (Newsome *et al.*, 2000b) mutants the labeling of R2-R5 axons revealed only a small number of axons that mistargeted into the medulla of third instar larva. Additionally, the labeling of R1-R6 axons in *trio* mutant adults revealed normal R-cell axon targeting to the lamina layer (Newsome *et al.*, 2000b). As *Pak* (Hing *et al.*, 1999), *rac* (Hakeda-Suzuki *et al.*, 2002), and *trio* (Newsome *et al.*, 2000b) are all required for R-cell axon guidance, the function of the Dock-Pak-Rac-Trio pathway is likely to promote proper R-cell guidance, but not R-cell axon targeting.

1.3 Specific molecules involved in *Drosophila* axonal fasciculation

1.3.1 Fas II

Fas II was initially identified as a potential adhesive molecule necessary for axonal fasciculation based on expression pattern detected on subsets of axon pathways in the grasshopper embryo (Bastiani *et al.*, 1987). Gremmingloh *et al.* (1991) demonstrated by immunohistochemistry using an anti-Fas II antibody that Fas II is expressed in the three longitudinal axon pathways located on either side of the *Drosophila* CNS midline: the inner vMP2; the middle MP1; and the outer FN3 tracts. Analysis of *fas II* mutant *Drosophila* embryos revealed the need for Fas II by CNS axons to properly fasciculate along normal pathways (Lin and Goodman, 1994). While *wt* MP1 and vMP2 axons normally follow pioneer dMP2 and pCC axons in the middle MP1 and inner vMP2 CNS axon tracts, in *fas II* mutants MP1 and vMP2 axons failed to fasciculate with dMP2 and pCC pioneer axons (Lin and Goodman, 1994). The MP1 and vMP2 axons of *fas II* mutants behaved as pioneer axons themselves by extending in the proper, general

direction without interacting with other axons. Therefore, the loss-of-*fas II* phenotype revealed a requirement of Fas II for the proper fasciculation of CNS axons that is independent from axonal targeting mechanisms.

Lin and Goodman (1994) observed a CNS Fas II GOF phenotype that is opposite to the *fas II* LOF phenotype when Fas II was expressed in *wt* CNS axonal tracts—at stage early-14 the vMP2 and MP1 pathways aberrantly fused together while at stage 16 the FN3 and MP1 pathways formed abnormal bundles. A similar adhesive GOF phenotype was observed for Fas II in the *Drosophila* embryonic motor nervous system. In each abdominal hemisegment of a *wt* embryo, motor axons exit the CNS in two main projections: the intersegmental nerve (ISN) and the segmental nerve (SN) (Araujo and Tear, 2003). Motor axons branch off of the ISN and SN projections at specific choice points: ISNb and ISNd defasciculate ventrally from the ISN, leaving the ISN to further extend and innervate dorsal muscles while the SNc defasciculates from the SN, leaving a bundle called the SNa to extend dorsally and innervate lateral muscles. The increased expression of Fas II by motor axons led to the failure of ISNb axons to defasciculate from the ISN and a misrouting of the SNa to aberrantly fasciculate with the ISN (Lin and Goodman, 1994). Together, these results demonstrate that Fas II acts to promote adhesion of different axonal subtypes as removal of *fas II* results in a failure of axons to remain fasciculated while an increase of Fas II dosage results in aberrant axonal fasciculation.

1.3.2 Beaten path

Beaten path (Beat) was identified in a genetic screen of EMS-induced mutants with defects in CNS axonal trajectories (Seeger *et al.*, 1993). Thus far, fourteen *beat*-like

genes have been identified in *Drosophila* (Pipes *et al.*, 2001). *beat-1a*, the first *beat* gene that was identified, encodes an immunoglobulin-containing protein that is secreted by motor axons (Bazan and Goodman 1997; Mushegian, 1997). In *beat-Ia* mutant embryos, ISNb and ISNd motor axons were found to remain fasciculated with the ISN projection while SNc failed to defasciculate from the SN (Fambrough and Goodman, 1996). These results indicate a requirement for Beat 1a for the proper defasciculation of motor axons at normal branch points. Fambrough and Goodman (1996) further demonstrated the anti-adhesive nature of *beat-1a* by examining double mutants for *fas II* and *beat-1a*. While 90% of abdominal segments in embryos double homozygous for a weak *fas II* allele and a null *beat-1a* allele demonstrated a strong *beat* phenotype, only 41% of segments homozygous for a strong hypomorphic *fas II* allele and a null *beat-1a* allele showed a strong *beat* phenotype. This result indicates that *beat-1a* can suppress the *fas II* defasciculation phenotype. The genetic interaction of *fas II* and *beat-1a* is unlikely to reflect physical interaction of Fas II and Beat-1a, as the ectopic expression of Beat-1a on CNS axonal pathways did not disrupt axonal fasciculation and cell co-aggregation experiments using sets of S2 cells that express either Fas II or membrane-bound Beat-1a did not demonstrate Beat-1a/Fas II interaction (Fambrough and Goodman, 1996). Rather, Beat-1a may act to promote defasciculation through another unknown receptor.

Beat-1a may act through competition for an unknown receptor with membrane-bound Beat-1c (Pipes *et al.*, 2001). In the embryonic motor nervous system, Pipes *et al.* (2001) found that Beat-1c is expressed in a subset of axonal projections and that loss-of-*beat-1c* can partially suppress the adhesive loss-of-*beat-1a* phenotype. This result supports a model in which an extending axon that expresses a Beat receptor may use the

receptor to fasciculate along a Beat-1c expressing pioneer axon. By secreting Beat-1a the adhesiveness of the follower axon to the pioneer is decreased, as Beat-1a would compete for the Beat receptor (Pipes *et al.*, 2001). The identification of the Beat receptor, the examination of the Beat receptor LOF phenotype, and the study of genetic interaction of the Beat receptor with Beat-1a and Beat-1c will be necessary to test this model of Beat function. Another molecule has been exhibited to have a role in the defasciculation of motor axons—the transmembrane Sema family member Sema1a (Yu *et al.*, 1998). Sema1a has been demonstrated to regulate axonal defasciculation through a number of downstream effectors including the receptors PlexinA (PlexA) (Winberg *et al.*, 1998) and Otk (Winberg *et al.*, 2001) and the flavoprotein monooxygenase Molecule Interacting with CasL (MICAL) (Terman *et al.*, 2002). The A kinase anchoring protein Nervy and protein kinase A (PKA) have been found to antagonize Sema1a signaling and are needed for motor axons to remain properly fasciculated (Terman and Kolodkin, 2004).

1.3.3 Sema1a, PlexA, Off-track, and MICAL

The Semaphorins are a family of both secreted and cell-surface molecules that all share a conserved 500 amino acid amino-terminal "Sema" domain (Nakamura *et al.*, 2000; Tamagnone and Comoglio, 2000; Castellani and Rougon, 2002; Pasterkamp and Kolodkin, 2003). The transmembrane protein Sema1a was originally chosen for study as a candidate for regulating *Drosophila* axonal fasciculation as antibody perturbation experiments had shown that grasshopper SemaI was required for the proper trajectory of Ti1 pioneer axons (Kolodkin *et al.*, 1992). The observation that a viral Semaphorin, vaccinia A39R could be used to affinity purify a Plexin receptor (Comeau *et al.*, 1998) led Winberg *et al.* (1998) to investigate the potential role for PlexA as a receptor for the

ligand Sema1a. As PlexA has no intrinsic kinase activity (Tamagnone *et al.*, 1999), but the mammalian PlexinsB1 and A3 were found to copurify with a tyrosine-phosphorylated protein of ~ 160 kDa, (Winberg *et al.*, 2001) studied the role of the 160 kDa receptor tyrosine kinase Otk in the Sema1a signaling. Kolodkin and colleagues isolated both MICAL (Terman *et al.*, 2002) and the A kinase anchoring protein Nervy (Terman and Kolodkin, 2004) as PlexA-interacting proteins using PlexA as a "bait" molecule in a Y 2-H screen of a *Drosophila* embryonic cDNA library. Sema1a, PlexA, Otk, MICAL, Nervy, and PKA are all expressed in the embryonic CNS, motor neurons, and the motor neuron axonal projections into the periphery and genetic and biochemical evidence suggests that together, these molecules act in biochemical pathways that regulate axonal defasciculation (Pulido *et al.*, 1992; Winberg *et al.*, 1998; Winberg *et al.*, 2001; Yu *et al.*, 1998; Terman *et al.*, 2002; Terman and Kolodkin, 2004).

Examination of the CNS and motor axon projections for *sema1a*, *plexA*, *otk*, and *MICAL* mutants has revealed similar LOF phenotypes, supporting the idea that these molecules all act in the same biochemical pathway (Winberg *et al.*, 1998, Winberg *et* al., 2001; Yu *et al.*, 1998; Terman *et al.*, 2002). Consistently, the third longitudinal FN3 tract of the *sema1a*, *plexA*, *otk*, or *MICAL* mutant CNS exhibited an abnormally discontinuous structure featuring individual axons that contact the medial MP1 pathway. A defect in the defasciculation of ISNb from the ISN motor axon tract, or in the defasciculation of motor axons from the ISNb to innervate target ventral muscles 6 and 7, or 12 and 13 was observed in *sema1a*, *plexA*, *otk*, and *MICAL* mutants. As well, the SNa axonal tract was frequently found to stall rather than to branch in a characteristic "pitchfork" pattern that normally results in proper innervation of muscles 22 and 23. Genetic interaction of

sema1a, *plexA*, *otk*, and *MICAL* was revealed by testing the phenotype of transheterozygotes for *sema1a/plexA* (Winberg *et al.*, 1998), *otk/plexA* and *otk/sema1a* (Winberg *et al.*, 2001), and *MICAL/plexA* and *MICAL/sema1a* (Terman *et al.*, 2002). In all cases, the examined transheterozygous individuals had phenotypes similar to the LOF phenotype characterized for each individual gene, supporting the idea that Sema1a, PlexA, Otk, and MICAL function in an activating manner in the same biochemical pathway. GOF studies were conducted to further demonstrate the relationship of *sema1a*, *plexA*, *otk*, and *MICAL* in the motor nervous system. Sema1a expression in the muscles of *wt* embryos revealed an enhancement of the bypass of ISNb axons at the normal ISNb branch point from the ISN tract (Yu *et al.*, 1998). The *sema1a* GOF phenotype was suppressed by removal of 50% dosage of either *plexA* (Winberg *et al.*, 1998), *otk* (Winberg *et al.*, 2001), or *MICAL* (Terman *et al.*, 2002) in the *sema1a* GOF background indicating that PlexA, Otk, and MICAL act downstream from Sema1a.

The molecular mechanism of Sema1a signaling in *Drosophila* motor axons is not fully understood. The observation that expression of a truncated Sema1a protein that lacks the normal transmembrane and cytoplasmic domains in motor neurons could partially rescue embryonic neuronal *sema1a* LOF phenotypes supports the idea that Sema1a acts as a ligand in motor neurons (Yu *et al.*, 1998) to activate signaling events through the downstream molecules PlexA and Otk (Winberg *et al.*, 2001). As PlexA and Otk genetically and physically interact with each other, the interaction of Sema1a and PlexA may allow the heterodimerization of PlexA and Otk to trigger downstream events in the growth cone (Winberg *et al.*, 2001). The interaction of Sema1a and PlexA may trigger the phosphorylation of PlexA and Otk because both PlexA (Tamagnone *et al.*,

1999) and Otk (Pulido *et al.*, 1992) have been shown to be tyrosine phosphorylated *in vitro*. The observation that *otk* has altered residues necessary for kinase activity (Winberg *et al.*, 2001) has led to the proposal that Otk is kinase-dead (Kroiher *et al.*, 2001) as is the receptor tyrosine kinase Derailed that is involved in CNS and mesoderm guidance events (Yoshikawa *et al.*, 2001). The identity of the tyrosine kinase that would phosphorylate PlexA and Otk following Sema1a activation is unknown. Proper motor axon defasciculation also requires MICAL downstream of PlexA and Otk (Terman *et al.*, 2002). The finding that expression of a mutant form of MICAL with a non-functional FAD fingerprint 1 motif was unable to rescue the loss-of-*MICAL* phenotype while expression of the same construct in a *wt* background was capable of behaving in a dominant-negative manner supports the idea that MICAL mediates the defasciculation of motor axons through the oxidization of an unknown substrate (Terman *et al.*, 2002).

The observation that both Sema1a and the Sema1a downstream receptor, PlexA are expressed along the length of motor axons suggests the existence of a mechanism to separate Sema1a from PlexA. As a follower axon fasciculates with a pioneer, Sema1a must be kept separate from PlexA otherwise the follower axon will be triggered to defasciculate from the pioneer axon prior to reaching a branch point. The isolation of Sema1a may be accomplished by the interaction of Sema1a with another unknown molecule that prevents unwanted Sema1a/PlexA interaction during axonal fasciculation. Once a follower axon reaches a branch point, a specific signal may instruct the unknown Sema1a-interacting molecule to release Sema1a and permit the Sema1a ligand to interact with the PlexA receptor. Alternatively, a similar regulatory molecule may bind to PlexA and thus prevent PlexA from binding to Sema1a prior to reaching a specific branch point

by a follower axon. The identification and genetic and biochemical analysis of a Sema1a or PlexA regulatory molecule will be necessary to understand how the molecules Sema1a and PlexA, expressed along the length of an axon, can direct defasciculation only at specific choice points.

1.3.4 Nervy and Protein kinase A

The A kinase anchoring protein Nervy was identified in a Y 2-H library screen using the PlexA cytoplasmic domain as a "bait" molecule (Terman and Kolodkin, 2004). Genetic analysis of Nervy and the associated cyclic adenosine monophosphate (cAMP)-dependent kinase PKA have revealed roles for these signaling molecules in the regulation of Sema1a-signalled motor and CNS axonal defasciculation (Terman and Kolodkin, 2004). The motor axons of both *nervy* and *pka* mutants exhibit a failure to remain fasciculated prior to reaching normal choice points. In *nervy* and *pka* mutants, abnormal defasciculation of motor axons was observed from the ISN, ISNb, and SNa pathways, leading to projection into incorrect muscle fibers. Additionally, CNS axons were observed to form loose connections within the third longitudinal FN3 tract and to inappropriately extend away from the CNS (Terman and Kolodkin, 2004). These results suggest that Nervy and PKA promote the fasciculation of motor and CNS axons.

The ability of Nervy and PKA to promote axonal fasciculation was found to antagonize the capacity of Sema1a signaling to permit axonal defasciculation. Mutants for *nervy* and *pka* were found to enhance the Sema1a GOF phenotype resulting from increased Sema1a expression in muscles, which is consistent with the idea that *nervy* and *pka* negatively regulate Sema1a axonal defasciculation (Terman and Kolodkin, 2004). Biochemical and genetic evidence has shown that Nervy and PKA must be associated for

proper function in motor axons. Nervy and PKA were found to be associated in the embryo, as PKA was found to immunoprecipitate PlexA from embryo lysate (Terman and Kolodkin, 2004). *In vivo*, Terman and Kolodkin (2004) demonstrated while expression of *wt* Nervy could rescue the *nervy* axonal mutant phenotype, the expression of mutant Nervy with a single amino acid substitution that disrupted Nervy/PKA interaction was unable to rescue the loss-of-*nervy* phenotype. This result indicates that Nervy must physically interact with PKA for proper function in motor axons.

Together, the data from Terman and Kolodkin (2004) indicates that Nervy directly interacts with PKA and may recruit PKA to the plasma membrane as Nervy can bind to the PlexA receptor. As PKA is a cAMP-dependant kinase, Nervy/PKA modulation of Sema1a signaling likely occurs in a cAMP-dependant fashion. In this manner, Sema1a signaling could be modulated by the adjustment of cAMP levels within the growth cone to respond to additional cues. A number of molecules have been identified that can modify cyclic nucleotide concentrations inside neurons, including the chemokine stromal cell-derived factor 1 (Xiang *et al.*, 2002; Chalasani *et al.*, 2003) and the G protein-coupled receptor adenosine A2b that has been shown to act as a Netrin-1 receptor (Corset *et al.*, 2000). By coupling Sema1a to cyclic nucleotide levels that are regulated by other receptors, the path of an extending growth cone can potentially be affected by a number of different guidance cues.

1.4 Rational for the current studies

1.4.1 A requirement for Off-track in layer-specific neuronal connectivity

The observation that the SH2/SH3 adapter protein Dock (Garrity *et al.*, 1996) and two receptor protein tyrosine phosphatases, LAR (Clandinin *et al.*, 2001) and PTP69D (Garrity *et al.*, 1999), are required for the proper termination of R1-6 growth cones led us to search for a receptor tyrosine kinase involved in the targeting of R1-R6 axons to the lamina layer of the *Drosophila* visual system. While clearly protein tyrosine phosphorylation is a key-signaling event in the regulation of R1-R6 lamina-specific targeting, no protein tyrosine kinase had been identified in this process. We chose to test the role of the receptor tyrosine kinase Otk in regulating proper R1-R6 axon targeting for two reasons. First, a P-element insertion in the 5' flanking region of the *otk* gene had been shown to cause a weak R-cell projection phenotype in our laboratory (Rao, Y., unpublished data). Second, Winberg *et al.* (2001) identified a role for Otk in the proper guidance of *Drosophila* motor axons. Chapter 2 of this thesis will elaborate upon our studies that examine the role of Otk in lamina-specific R1-R6 axonal targeting.

1.4.2 Sema1a can function as a receptor to promote axonal fasciculation

Previous work had indicated that Sema1a acts as an upstream activating ligand of the Otk receptor necessary for the defasciculation of motor axons at specific choice points in *Drosophila* (Winberg *et al.*, 2001). In order to test whether Sema1a acted in a similar manner in the *Drosophila* visual system, we compared the LOF phenotypes of both *otk* and *sema1a* (Cafferty *et al.*, 2004). Surprisingly, we found the *sema1a* mutant phenotype was different from the *otk* LOF phenotype. Further analysis of Sema1a has revealed a role for Sema1a in the promotion of R-cell axonal fasciculation by acting as a

receptor. Chapter 3 of this thesis will describe the attractive role of Sema1a as a guidance receptor in the development of the *Drosophila* visual system.

Chapter 2

The organization of neuronal connections into specific layers within the nervous system is a theme common to both vertebrates and invertebrates. In the *Drosophila* visual system, tyrosine phosphorylation has been demonstrated as being a key-signaling event in specifying the termination of R1-R6 axons at the lamina layer in the optic lobe. While two protein tyrosine phosphatases, PTP69D and LAR, and an SH2/SH3 adapter protein, Dock, have been shown as necessary for the proper termination of R1-6 growth cones at the lamina, the receptor tyrosine kinase involved in the lamina-specific targeting of R1-R6 axons was unknown. In this chapter, we describe a requirement for the receptor tyrosine kinase Otk for the layer-specific targeting of R1-R6 axons in the lamina layer.

The Receptor Tyrosine Kinase Off-track Is Required for Layer-specific Neuronal Connectivity in Drosophila

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Summary

The nervous system in many species consists of multiple neuronal cell layers, each forming specific connections with neurons in other layers or other regions of the brain. How layer-specific connectivity is established during development remains largely unknown. In the Drosophila adult visual system, photoreceptor (R-cell) axons innervate one of two optic ganglia layers; R1-R6 axons connect to the lamina layer, while R7 and R8 axons project through the lamina into the deeper medulla layer. Here, we show that the receptor tyrosine kinase Off-track (Otk) is specifically required for lamina-specific targeting of R1-R6 axons. Otk is highly expressed on R1-R6 growth cones. In the absence of *otk*, many R1-R6 axons connect abnormally to medulla instead of innervating the lamina. We propose that Otk is a receptor or a component of a receptor complex that recognizes a target-derived signal for R1-R6 axons to innervate the lamina layer.

Introduction

The vertebrate central nervous system has a multi-layered architecture in which different neuronal cell layers receive innervation from axons that project from distinct neuronal populations. To establish such layer-specific neuronal connections, it is thought that each neuronal cell layer must express specific membrane-bound surface labels, which are then recognized by specific receptors expressed on the growth cone of incoming axons (Bolz and Castellani, 1997). Due to the complexity of the vertebrate nervous system, however, it is only recently that the molecular mechanism underlying the formation of layer-specific connectivity has begun to be elucidated. For instance, it has been shown that the expression of the cell adhesion molecule N-Cadherin by subtypes of laminae in the chick visual system is necessary for layer-specific targeting of distinct subtypes of retinal axons in the optic tectum (Inoue and Sanes, 1997). Recent studies also implicate a role for Ephs and their ligands Ephrins in regulating the formation of layer-specific thalamocortical connections in mice (Mann et al., 2002).

The formation of photoreceptor-to-optic lobe connections in the Drosophila adult visual system is an excellent and simple model to study the molecular mechanisms that control the establishment of layer-specific neuronal connectivity during development (Clandinin and Zipursky, 2002; Tayler and Garrity, 2003). The Drosophila adult visual system is comprised of the compound eye and the optic lobe. The compound eye consists of ~800 ommatidia or single eye units, each containing eight different photoreceptor cells (R-cells). R-cells project axons into one of two optic ganglion layers in the brain. R1-R6 cells connect to the superficial layer of the optic lobe, the lamina, and are responsible for the absorption in the green range. While R7 and R8 cells connect to the deeper medulla

layer, and are responsible for the absorption in the ultraviolet and blue range. The formation of layer-specific R-cell connection pattern begins at the third-instar larval stage. Precursor cells in the third-instar larval eye-imaginal disc begin to differentiate into R-cells. Within each ommatidium, the R8 precursor cell differentiates first and projects its axon through the optic stalk and the developing lamina into the medulla. Axons from the later differentiated R1-R7 cells within the same ommatidium form a single bundle with the pioneer R8 axon until they encounter a layer of glial cells (i.e. marginal glia) within the lamina layer. There they have to make a binary choice; either stop or keep going into the medulla. The R1-R6 growth cones terminate within the lamina in response to an unknown stop signal from lamina glial cells (Poeck et al., 2001), their intermediate target at larval stage. In contrast, R7 growth cones extend further to join R8 growth cones in the medulla. During pupation, R1-R6 growth cones undergo further stereotyped rearrangements and subsequently form synaptic connections with lamina neurons (Clandinin and Zipursky, 2000; Meinertzhagen and Hanson, 1993).

Recent studies have identified several cell surface proteins that are required for R-cell connectivity. Specifically, N-Cadherin, the receptor tyrosine phosphatase Lar and the Cadherin-related protein Flamingo have each been shown to be required for the establishment of local synaptic connections between R1-R6 axons and lamina cartridge neurons (Clandinin et al., 2001; Lee et al., 2001; Lee et al., 2003). An additional role for N-Cadherin, Lar and the receptor tyrosine phosphatase PTP69D in R7 axons and Flamingo in R8 axons for forming local connections with target cells within the medulla has also been reveled (Clandinin et al., 2001; Lee et al., 2003; Maurel-Zaffran et al., 2001; Newsome et al., 2000a; Senti et al., 2003). However, loss of N-Cadherin or

Flamingo does not affect the initial choice between lamina versus medulla target selection. In their absence R1-R6 still connect to the lamina, while R7 and R8 still choose the medulla for establishing synaptic connections. While loss of *Ptp69D* or *Lar* does affect the initial projections of R1-R6 axons (Clandinin et al., 2001; Garrity et al., 1999), the completed pattern of lamina-versus-medulla target selection in adult *Ptp69D* or *Lar* mutants remains largely unchanged (Clandinin et al., 2001; Newsome et al., 2000a). These data argue against a direct role for either PTP69D or Lar in specifying lamina-specific targeting of R1-R6 axons. In addition to the above cell surface receptors, two Drosophila receptor tyrosine kinases, the insulin receptor and Eph receptor, are also required for regulating different aspects of R-cell axon guidance (Dearborn et al., 2002; Song et al., 2003). However, neither has been shown to play a role in regulating layer-specific R-cell connectivity. Thus, it remains unclear how R-cell axons detect layer-specific targeting signals to make the binary decision for choosing either lamina or medulla to establish synaptic connections.

In a search for genes that are required for R-cell projections in the developing visual system, we have identified the receptor tyrosine kinase Otk as a key determinant in specifying the binary lamina versus medulla target selection. While Otk was originally isolated based on its homology with the *trk* family of neurotrophin receptors in vertebrates (Pulido et al., 1992), more recent studies suggest strongly that Otk is not a homologue of the vertebrate Trk A receptor (Kroiher et al., 2001). It has been shown that in vitro Otk mediates cell-cell adhesion in a Ca^{2+}-independent homophilic manner (Pulido et al., 1992), while in vivo it functions downstream of Semaphorin-1a (Sema-1a) to regulate motor axon guidance at the embryonic stage (Winberg et al., 2001). In this

study, we show that Otk is predominantly localized to R1-R6 growth cones in the fly visual system and is specifically required for lamina-specific targeting of R1-R6 axons. We propose that Otk recognizes a lamina-derived signal for R1-R6 targeting.

Materials and methods

Genetics

otk$^{EP(2)2017}$ was obtained from the Berkeley *Drosophila* Genome Project. *sema*^{P1} was provided by Alex Kolodkin. *otk*^{3}, UAS-*otk* and UAS-*fasII* were provided by Cory Goodman. Large clones of *otk*^{3} or *sema*^{P1} were generated in an otherwise heterozygous or wild type eye by eye-specific mitotic recombination using the eyFLP/FRT system (Newsome et al., 2000a). Using this method, ~80-90% of ommatidia in a mosaic eye were *otk*^{3} or *sema*^{P1} mutant cells in all individuals examined. Since mitotic recombination is under control of the eye-specific eyFLP, mutant clones were generated in the eye but not in the target region, thus allowing us to determine if *otk* is required in the eye for regulating R-cell connectivity. Rescue experiments were performed by crossing a GMR-GAL4 and a UAS-*otk* transgene into the *otk*$^{EP(2)2017}$/ *otk*^{3} transheterozygous individual, which allows the eye-specific expression of the *otk* transgene in *otk*$^{EP(2)2017}$/ *otk*^{3} mutants. The potential effect of the *otk* mutation on R7 projections was examined by crossing the adult R7 marker PANR7-GAL4::UAS-Synaptobrevin-GFP into *otk*^{3} mosaics as described (Lee et al., 2001). To express Otk in R7 axons, UAS-*otk* flies were crossed with PM181-GAL4, UAS-*lacZ* flies.

Histology and Immunohistochemistry

Adult retinae was dissected, fixed, and embedded in plastic for tangential sectioning as described (Tomlinson and Ready, 1987). Cryostat sections of adult mosaic heads were stained with mAb 24B10 or anti-β-galactosidase antibody as described (Garrity et al., 1996). Eye-brain complexes from third-instar larvae were dissected and

stained with antibodies as described (Ruan et al., 1999). Antibodies to Chaoptin (24B10) (1:100 dilution, DSHB), Prospero (1:200 dilution, DSHB), Boss (1:2000 dilution), Repo (1:10 dilution, DSHB), Otk (1:100 dilution) (Pulido et al., 1992), GFP (1:1000 dilution, Molecular Probes) and β-galactosidase (1:100 dilution) were used as primary antibodies. For HRP/DAB visualization, HRP-conjugated anti-mouse and anti-rabbit secondary antibody was used at 1:200 dilution. For fluorescent staining, texas-red- or FITC-conjugated goat anti-rabbit and anti-mouse secondary antibody (Jackson Immunochemicals) was used at 1:200 dilution. Epifluorescent images were captured using a high-resolution fluorescence imaging system (Canberra Packard) and analyzed by 2D Deconvolution using MetaMorph imaging software (Universal Imaging, Brandywine, PA).

The percentage of mistargeted R2-R5 axons or axon bundles in the medulla in *otk* and *sema* mutants was estimated by following the method described previously (Garrity et al., 1999) with only minor modification. Since mistargeted R2-R5 axons were observed in individuals that were much younger than that reported previously (Garrity et al., 1999), the mean number of ommatidial rows were subtracted by 4 instead of 9 ommatidial rows. R-cell axons projected from these subtracted younger ommatidial rows presumably had not reached the brain.

Results

otk Is Required for R-cell Growth-cone Targeting

To identify genes that are required for layer-specific targeting of R-cell axons, we examined R-cell projection pattern in available mutants, including novel P-element insertions from the Berkeley Drosophila Genome Project as well as mutations that disrupt known genes that are expressed specifically in the nervous system. Among them, we found that mutations in the *otk* gene caused a specific R-cell projection phenotype. Since the null mutation otk^3 in which the putative translational start codon and part of the signal peptide is deleted causes embryonic lethality (Winberg et al., 2001), we performed genetic mosaic analysis to examine axonal projections from otk^3 mutant R-cell clones. *otk* homozygous mutant tissues were generated in an otherwise heterozygous or wild-type eye-imaginal discs by eye-specific mitotic recombination using the eyFLP/FRT system (Newsome et al., 2000a). By examining mutant clones in adult mosaic eyes, we estimated that ~80-90% of ommatidia in each mosaic eye examined were *otk* mutant clones, which was consistent with the absence of most anti-Otk immunoreactivity in the lamina in all otk^3 mosaic third-instar eye-brain complexes examined (see below).

The R-cell projection pattern in *otk* mosaic larvae was examined using the monoclonal antibody 24B10 that visualizes all R-cell axons in the developing optic lobe (Van Vactor et al., 1988). In wild type (Fig. 1A), R1-R6 growth cones terminated within the lamina and then expanded significantly in size, which were seen as a continuous layer of 24B10 immunoreactivity within the lamina. Whereas expanded R7 and R8 growth cones form a highly organized pattern within the medulla. In otk^3 mosaic individuals (n=25 hemispheres, Fig. 1B), small gaps were frequently observed in the R1-R6 terminal

field. The terminal field within the medulla was also disorganized as thicker bundles were frequently observed within the medulla. Unlike some known mutations (e.g. *dock* and *pak*) that affect R-cell guidance (Garrity et al., 1996; Hing et al., 1999), loss of *otk* did not cause an obvious defect in the overall organization of R-cell axons within the developing optic lobe. The formation of topographic map also appeared normal.

To determine if mistargeting of R1-R6 axons causes the above defect, we used the larval R2-R5 marker *ro-τ-lacZ* to assess the initial targeting of a subset of R1-R6 axons at the third-instar larval stage. In wild type (Fig. 1C), the vast majority of R2-R5 axons stop within the lamina, and only a few labelled axons (average 3 mistargeted axons or axon bundles per hemisphere, n=19 hemispheres) projected into the medulla. In otk^{3} mosaic individuals (Fig. 1D), however, more than 32% (average 33 axons or axon bundles per hemisphere, n=16 hemispheres) of ommatidia projected one or more R2-R5 axons or axon bundles aberrantly into the medulla. A similar mistargeting phenotype (average 18 mistargeted R2-R5 axons or axon bundles per hemisphere, n=19 hemispheres) was also observed in $otk^{3}/otk^{EP(2)2017}$ transheterozygous larvae (Fig. 1E). However, the phenotype was less severe than that in otk^{3} mosaic larvae, which was likely due to the hypomorphic nature of the $otk^{EP(2)2017}$ allele. To further determine if the above phenotype was indeed due to the lesion in the *otk* gene locus, we performed transgene rescue experiments. Indeed we found that eye-specific expression of an *otk* transgene completely rescued the R1-R6 mistargeting phenotype in *otk* mutants (Fig. 1F). The average number of mistargeted R2-R5 axons or axon bundles was reduced to 3 in $otk^{3}/otk^{EP(2)2017}$ transheterozygous larvae expressing the *otk* transgene, which is similar to that in wild type. This result, taken together with that from eye-specific genetic mosaic analysis,

indicate that Otk is required in the eye for lamina-specific targeting of R1-R6 growth cones.

otk Is Expressed in the Developing Drosophila Adult Visual System

Previous studies demonstrated that Otk is specifically expressed in the nervous system at the embryonic stage (Pulido et al., 1992; Winberg et al., 2001). To determine if Otk is also expressed in the developing adult visual system at the larval stage, we stained third-instar larval eye-brain complexes with an affinity purified anti-Otk antibody (Pulido et al., 1992). In wild type (Fig. 2B, C), anti-Otk staining was detected on R-cell axons in the developing optic lobe. In the lamina, the staining overlapped largely with 24B10 immunoreactivity that reflects the expression pattern of Chaoptin, a cell surface adhesion molecule expressed exclusively on all R-cells and their axons (Van Vactor et al., 1988). The strongest staining was observed in the lamina plexus, comprised primarily of R1-R6 growth cones. Although anti-Otk immunoreactivity was also detected in the developing medulla, we could not tell if Otk is present on R7 and R8 growth cones due to the uniform staining pattern in the medulla neuropil that consists of both R-cell and non-R-cell axons (Fig. 2B,C). The specificity of anti-Otk staining was supported by the observation that the staining within the lamina was largely absent in otk^3 mosaic larvae (Fig. 2E, F). We conclude that Otk is expressed in developing R-cells and is localized predominantly to R1-R6 growth cones.

R-cell Differentiation and Fate Determination Occur Normally in *otk* Mutants

The R1-R6 mistargeting phenotype may reflect a direct role for Otk in regulating R-cell growth-cone targeting. Alternatively, the defect might be caused by abnormal R-cell differentiation or cell fate determination, for instance, the transformation of a R1-R6 cell into a R7 or R8 fate. To distinguish among those possibilities, we examined R-cell development by using R-cell specific developmental markers. Differentiating R7 and R8 cells in the developing eye disc were identified with anti-Prospero and anti-Boss antibodies, respectively. As in wild type (Fig. 3A, C), only one R7 (100%, n=2052 ommatidia in 13 eye discs, Fig. 3B) and one R8 (100%, n=3862 ommatidia in 16 eye discs, Fig. 3D) were observed in each ommatidium in all otk^3 mosaic eye discs examined. Consistently, examination of *otk* adult mosaic eyes did not reveal any defect in either the number or the organization of R-cells in all otk^3 mutant ommatidia examined (n=978 ommatidia in 10 eyes) (compare Fig. 3F to 3E). Thus, *otk* is not required for R-cell differentiation and cell fate determination.

***otk* Is Not Required for the Differentiation and Migration of Lamina Glial Cells**

Previous studies demonstrate a dynamic interaction between R-cell axons and lamina glial cells, the intermediate target of R1-R6 axons at larval stage (Poeck et al., 2001; Suh et al., 2002). On one hand, lamina glial cells produce an unknown stop signal to induce the initial termination of R1-R6 growth cones within the lamina (Poeck et al., 2001). On the other hand, R-cell axons also produce an unknown signal to induce the migration of lamina glial cells into the R1-R6 target region (Suh et al., 2002). To determine if the expression of Otk in R-cell axons is necessary for lamina glial cell

differentiation and/or migration, we examined the development of lamina glial cells in *otk* mutants. Glial cells were visualized using a monoclonal antibody that recognizes the glia-specific nuclear protein Repo. In wild type (Fig. 4A), R1-R6 axons stop in the lamina and expand their growth cones in between two layers of lamina glial cells (i.e. epithelial and marginal glia). Although lamina glial cells in *otk* mutants appeared less organized than that in wild type (compare Fig. 4B to 4A), the number of lamina glial cells surrounding the lamina plexus in *otk* mutants were similar to that in wild type (n=12 hemispheres), indicating that the migration of lamina glial cells occurred normally in *otk* mutants.

The Function of Otk in R-cell Growth Cones Appears to Be Independent of Sema-1a Signaling

Previous studies showed that Otk interacts with Plexin A in mediating a Sema-1a-induced repulsive response during motor axon guidance at embryonic stage (Winberg et al., 2001), raising the possibility that the role of Otk in R1-R6 growth cones is also dependent on Sema-1a signaling. If so, one would predict that loss of Plexin A or Sema-1a should cause a similar R1-R6 targeting phenotype. Unfortunately, we were unable to assess the role of *plexin A* during R1-R6 growth-cone targeting as the available *plexin A* mutation causes early lethality. And the *plexin A* gene is located on the fourth chromosome and thus not amenable to FLP/FRT-mediated mosaic analysis. However, we were able to examine R-cell projections in both *sema-la* homozygous null mutants (i.e. $sema^{P1}$) and *sema-la* eye-specific mosaic animals in which large clones of $sema^{P1}$ mutant eye tissues were generated similarly using the eyFLP/FRT system as described above.

Labelling of R-cell axons with MAb 24B10 staining revealed an R-cell projection phenotype in both *sema*^{P1} homozygous mutant and mosaic larvae. The R1-R6 terminal field in the lamina was severely disrupted, clumps and loop-like structures were frequently observed in *sema* mutants (Fig. 5C). In comparison, *otk* mutations caused only relatively mild defects in the organization of R-cell axons within the lamina (Fig. 5B).

To specifically assess the potential effect of *sema-1a* mutations on R1-R6 targeting, we used the *ro-τ-lacZ* marker to label R2-R5 axons in *sema*^{P1} homozygous mutant larvae. Surprisingly, although the organization of R-cell axons within the lamina was severely disrupted in *sema*^{P1} mutants (Fig. 5C), lamina-specific targeting of R2-R5 axons occurred in a largely normal fashion (Fig. 5F). In *sema*^{P1} homozygous mutants, the average number of mistargeted axons or axon bundles in each hemisphere is 7 (n=15 hemispheres), a few more than that in wild type (i.e. 3, Fig. 5D), but much less than that in *otk*^{3} mosaic animals (i.e. 33, Fig. 5E). Those observations argue against the possibility that Otk is regulated by Sema-1a for targeting R1-R6 axons to the lamina.

Loss of *otk* Severely Disrupts the Completed Pattern of R-cell Connectivity in Adult Flies

To determine the effect of the *otk* mutation on the completed pattern of R-cell-to-brain connectivity in adults, we examined R-cell axonal projections in *otk* mosaic heads. Again, large clones of *otk*^{3} mutant tissues were generated in the compound eye by eye-specific mitotic recombination. The completed R-cell projection pattern in adults was examined by staining frozen sections of *otk* mosaic heads with MAb 24B10. Although R-cell axons appeared to project into correct topographic locations, an increase in the

number of axon terminals within the medulla was observed in all sections examined (n=16 hemispheres) (compare Fig. 6B to 6A, 6D to 6C), suggesting that many mistargeted R1-R6 axons remained within the medulla.

To confirm this, we specifically labelled R1-R6 axons using an adult R1-R6 marker *Rh1-lacZ*. To accurately count the total number of axons that project abnormally into the medulla, we performed whole-mount staining of the brain instead of staining frozen sections. In wild type (Fig. 6E), all *Rh1-lacZ*-labeled axons connected to the lamina. In all 11 wild-type hemispheres examined, no labelled axons projected into medulla. In *otk* mosaic heads (Fig. 6F), however, a large number of R1-R6 axons were present in the medulla in *otk* mosaic animals (16 out of 17 hemispheres). Among 16 *otk* mosaic hemispheres that displayed mistargeting phenotype, 13 hemispheres were mounted properly such that the total number of mistargeted R1-R6 axons or axon bundles could be accurately counted. The average number of mistargeted R1-R6 axons or axon bundles per hemisphere was 336 (ranging from 119 to 363 in different hemispheres). Mistargeted axons were distributed evenly within the medulla. By dividing the average number of mistargeted R1-R6 axons or axon bundles (i.e. 336) with 800 (the approximately total number of ommatidial fascicles within an adult eye), we estimate that approximately 42% of ommatidia projected one or more R1-R6 axons aberrantly into the medulla. This is in marked contrast to that in *Ptp69D* adult mutants in which only a few mistargeted R1-R6 axon bundles (<5%) were observed within the medulla (Newsome et al., 2000a).

otk Is Not Required for R7 Axon Targeting

To determine if loss of *otk* also affects the targeting of other R-cells, we used the adult R7 marker PanR7-GAL4::UAS-Synaptobrevin-GFP to specifically assess the projections of R7 axons in *otk* adult mosaic heads in which the vast majority of R-cells are *otk* mutant cells. In wild type (Fig. 7A), R7 axons projected into a region (i.e. M6 layer) in the medulla that is deeper than the R8 terminal field (i.e. M3 layer). In all sections examined (10 hemispheres), we found that labelled R7 axons still projected into the correct locations within the medulla (Fig. 7B). Thus, unlike loss of *Ptp69D* or *Lar* (Clandinin et al., 2001; Maurel-Zaffran et al., 2001; Newsome et al., 2000a), mutations in *otk* do not affect R7 targeting.

The Expression of Otk in R7 Axons Was Not Sufficient for Targeting R7 Axons to Lamina

In our previous studies (Ruan et al., 2002), we showed that the expression of the Ste20-like ser/thr kinase Misshapen (Msn) or the cytoskeletal regulator Bifocal (Bif) in R7 cells under control of a larval R7-specific driver PM181-GAL4 caused some R7 growth cones to target into the lamina. To determine if the expression of Otk alone is sufficient for specifying lamina-specific targeting of R-cell axons, we examined the effect of expressing Otk in R7 axons using the PM181-GAL4 driver. In wild type (Fig. 8A), all labelled R7 axons projected through the lamina and terminated within the medulla. In all larvae expressing Otk in R7 cells (n=11 hemispheres), R7 axons still extended normally into the medulla (Fig. 8B).

Figures

Figure 1. The effect of *otk* mutations on R-cell projection pattern at larval stage. All R-cell axons in third-instar larvae (A and B) were stained with MAb 24B10. R2-R5 axons in third-instar larvae (C-F) were labeled with the larval R2-R5 marker *ro-τ-lacZ*. In wild type (A), after exiting the optic stalk (os), R7 and R8 growth cones passed through the lamina into the medulla, whereas R1-R6 growth cones stop within the lamina, which could be identified as a continuous line of MAb 24B10 immunoreactivity. In B, an otk^3 mosaic individual in which ~80-90% eye tissues were homozygous otk^3 mutant ommatidia, displayed defects in R-cell projections. The lamina plexus was uneven with the presence of small gaps. Abnormal thicker bundles were observed within the medulla. In wild type (C), *ro-τ-lacZ* labeled R2-R5 axons terminated within the lamina. In an otk^3 mosaic individual (D), many labeled R2-R5 axons projected aberrantly into the medulla. Similar mistargeting phenotype was also observed in $otk^3/otk^{EP(2)2017}$ transheterozygous larvae (E). In an $otk^3/otk^{EP(2)2017}$ transheterozygous larvae expressing an UAS-*otk* transgene in R-cells under control of the GMR-GAL4 driver (Mismer and Rubin, 1987) (F), most labeled R2-R5 axons now terminated within the lamina. Scale bar, 20 μm.

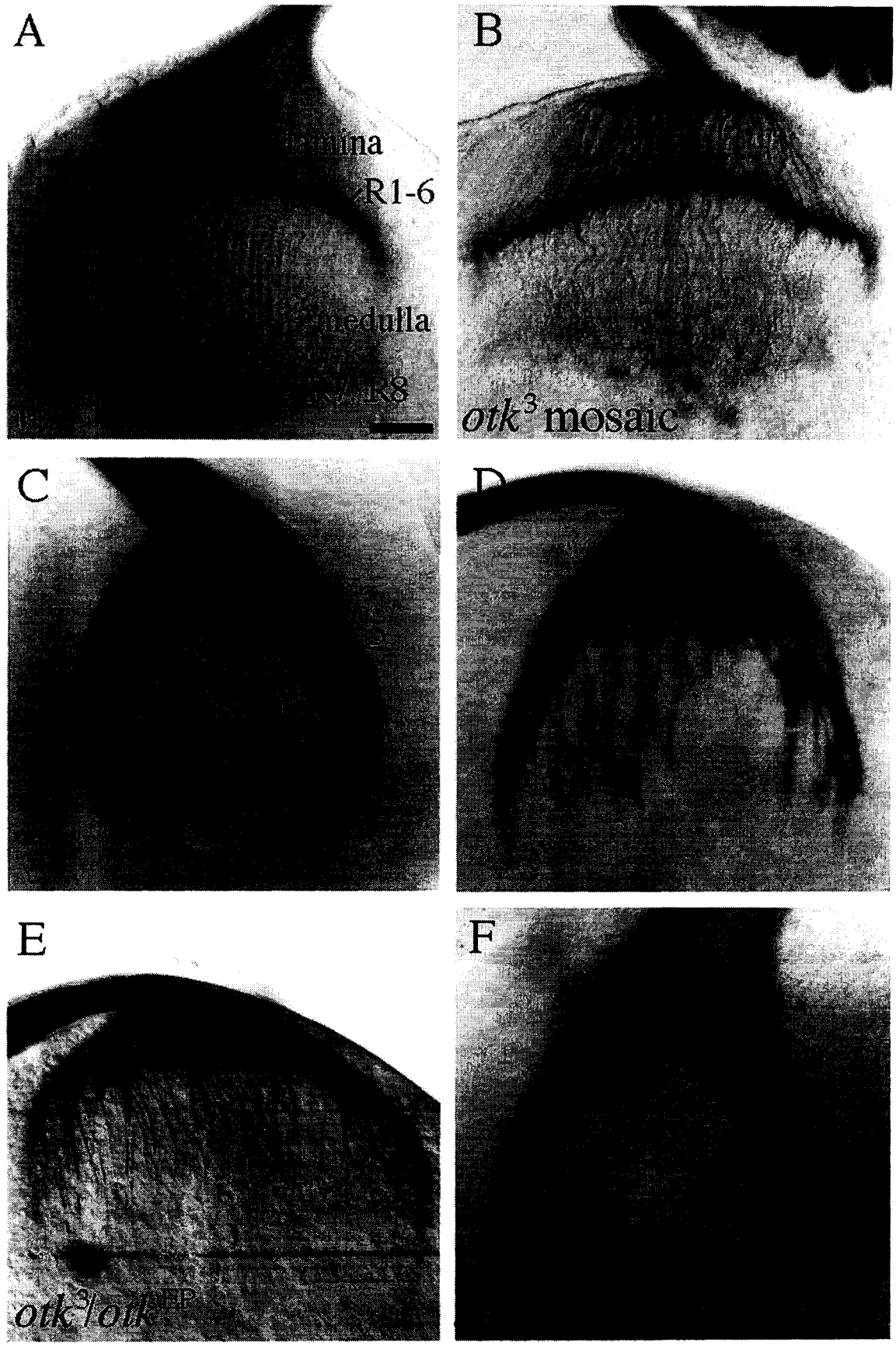

Figure 1

Figure 2. Otk is highly enriched in R1-R6 growth cones

Third-instar eye-brain complexes were double-stained with MAb 24B10 (green) and anti-Otk antibody (red). In a wild type optic lobe (A-C), anti-Otk immunoreactivity was detected in both lamina and medulla. The strongest staining was detected in the lamina plexus consisting mainly of R1-R6 growth cones at this developmental stage. In an otk^3 eye-specific mosaic individual in which most R-cell axons were otk^3 mutant axons (D-F), anti-Otk immunoreactivity was largely absent in the lamina plexus. Scale bar, 20 μm.

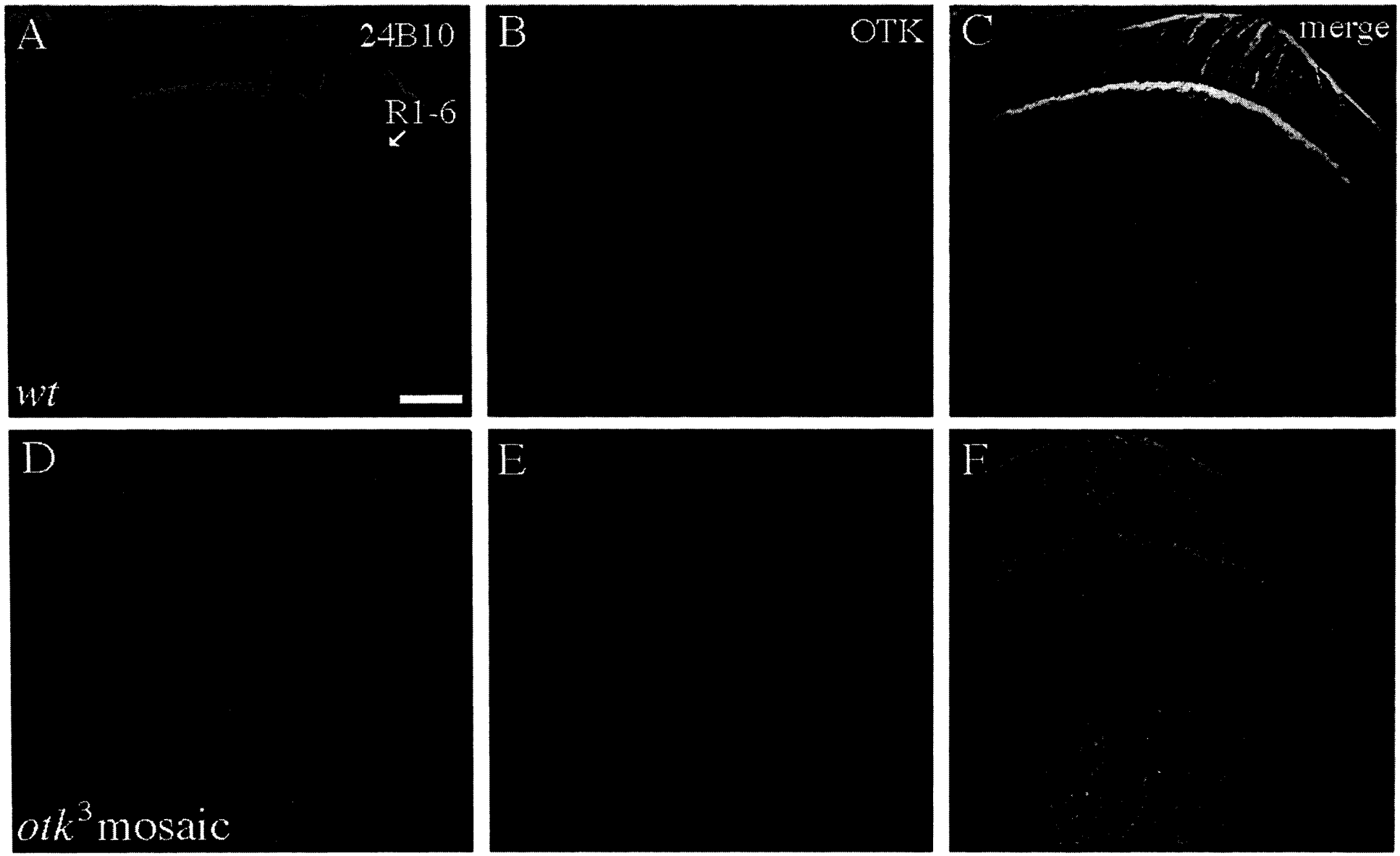

Figure 2

Figure 3. *otk* is not required for R-cell differentiation and cell fate determination. Third-instar eye-imaginal discs were stained with anti-Prospero (A and B) or anti-Boss antibody (C and D). In wild type, each ommatidium only contains a single R7 (a R7 nuclei indicated by arrow in A) and a single R8 cell (cell apical surface indicated by arrow in C). In an otk^3 mosaic eye-imaginal disc, only one R7 (B) and one R8 (D) were present in each ommatidium. Tangential sections of wild-type (E) and otk^3 adult mosaic eyes (F) did not reveal any defect in either the number or the organization of R-cells within each ommatidium. The arrow in F indicates a wild-type ommatidium surrounded by dense pigment granules. The arrowhead in F indicates an otk^3 mutant ommatidium that can be recognized by the absence of pigment granules. Scale bar, 10 μm.

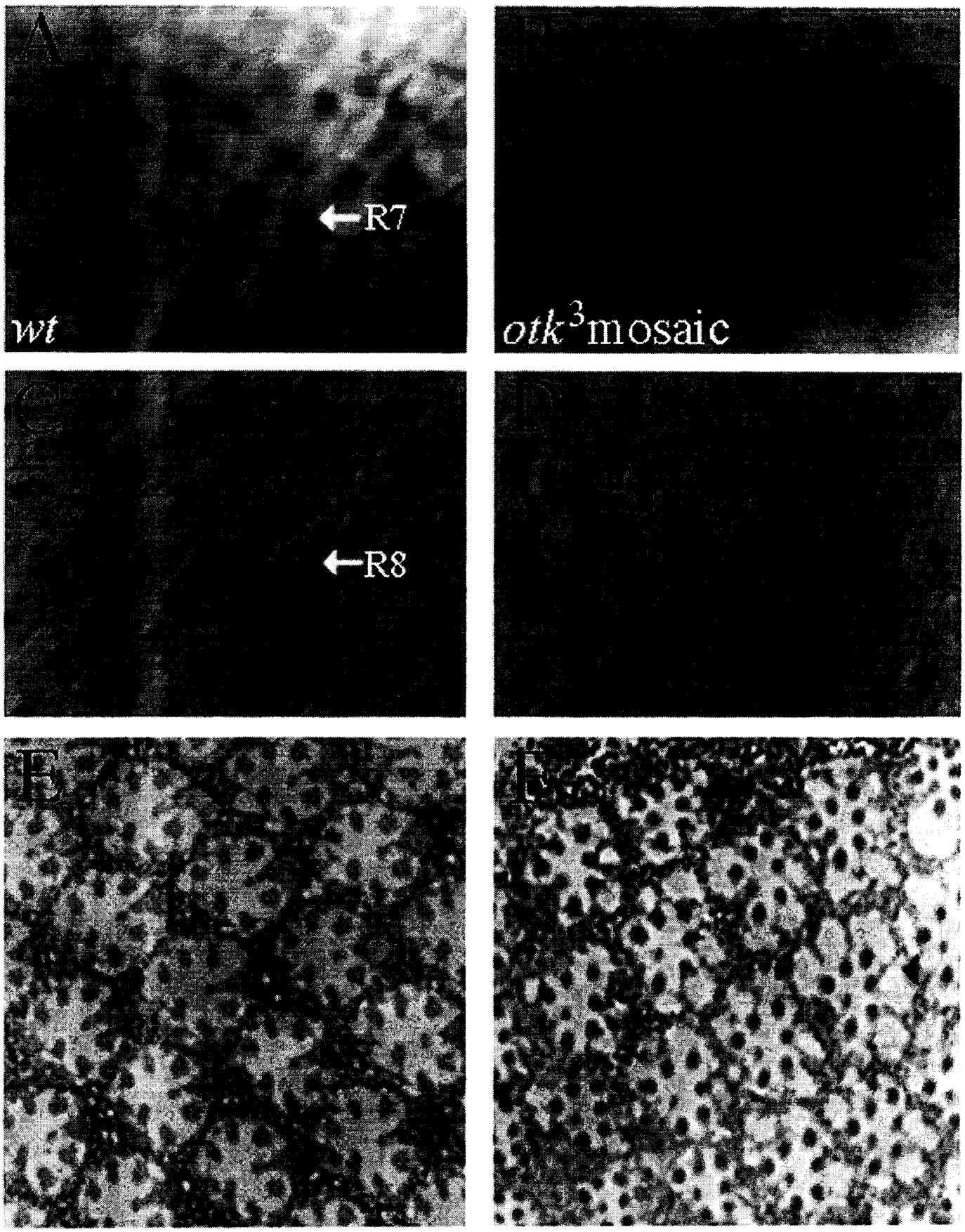

Figure 3

Figure 4. Lamina glial cell migration occurs normally in *otk*^{3} mutants.

Third-instar eye-brain complexes were double-stained with anti-Repo (red) and anti-β-galactosidase antibody (green). Anti-β-galactosidase antibody was used to visualized all R-cell axons in both wild type and *otk*^{3} mosaic individuals that carry a *glass-lacZ* transgene in which the expression of *lacZ* is under control of the eye-specific *glass* promoter (Mismer and Rubin, 1987). Anti-Repo recognizes the nuclear protein Repo expressed in all types of glial cells. In wild type (A), all R1-R6 growth cones (green) stop in between two layers of glial cells (red), epithelial (eg) and marginal glia (mg), forming the lamina plexus (lp). In an *otk*^{3} mosaic individual (B), lamina glial cells were still present at the R1-R6 termination site. Scale bar, 20 μm.

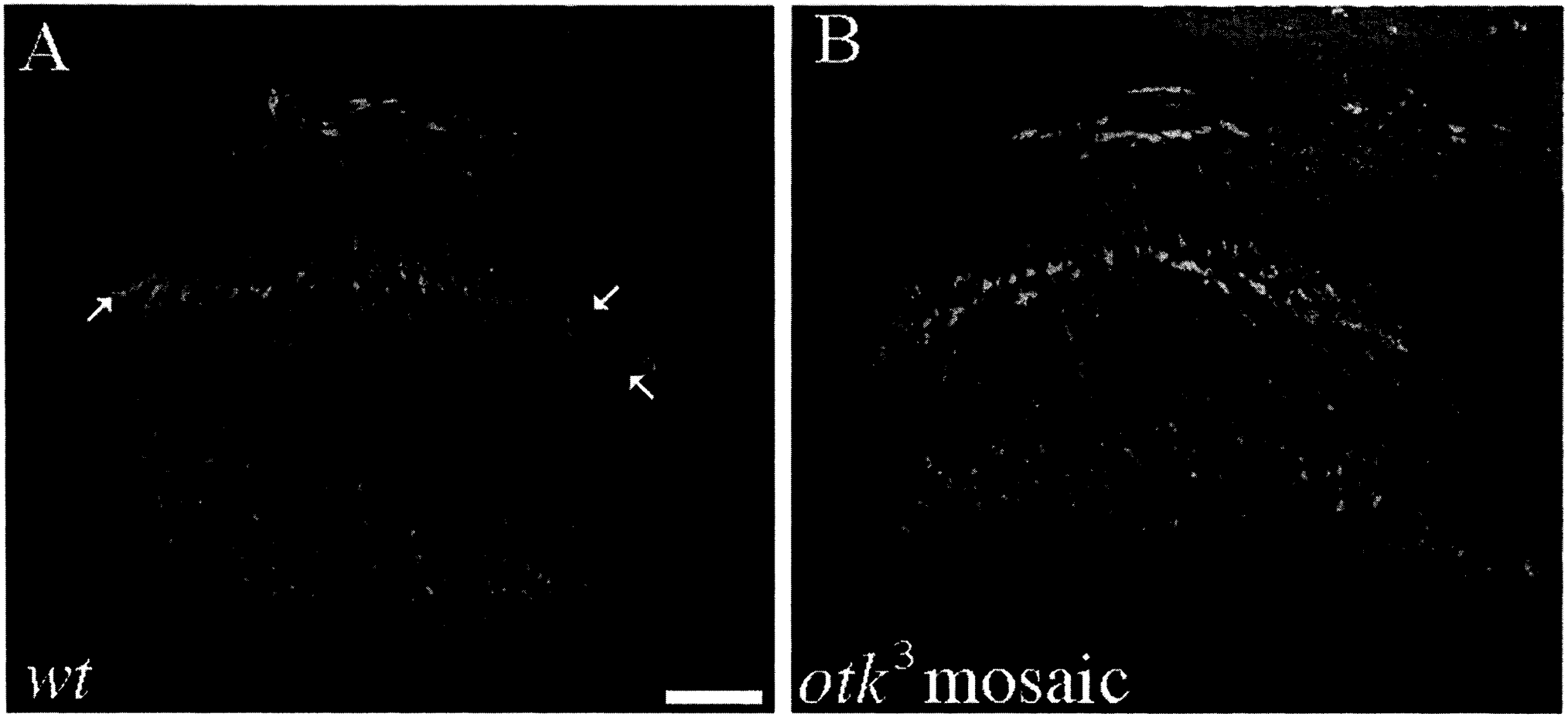

Figure 4

Figure 5. R1-R6 targeting appears largely normal in *sema* mutants.

Third-instar eye-brain complexes of wild type (A and D), *otk*^{3} eye-specific mosaic (B and E), *sema-1a*^{P1} eye-specific mosaic (C) and homozygous mutants (F) were stained with MAb 24B10 (A-C) or with anti-β-galactosidase antibody (D-F). Individuals in D-F carried the *ro-τ-lacZ* marker that labels R2-R5 axons at larval stage. Although *sema-1a*^{P1} caused a defect in the organization of R-cell axons within the lamina (C) that was more severe than that caused by the *otk*^{3} mutation (B), it did not significantly affect R1-R6 targeting (compare F to E). Scale bar, 20 μm.

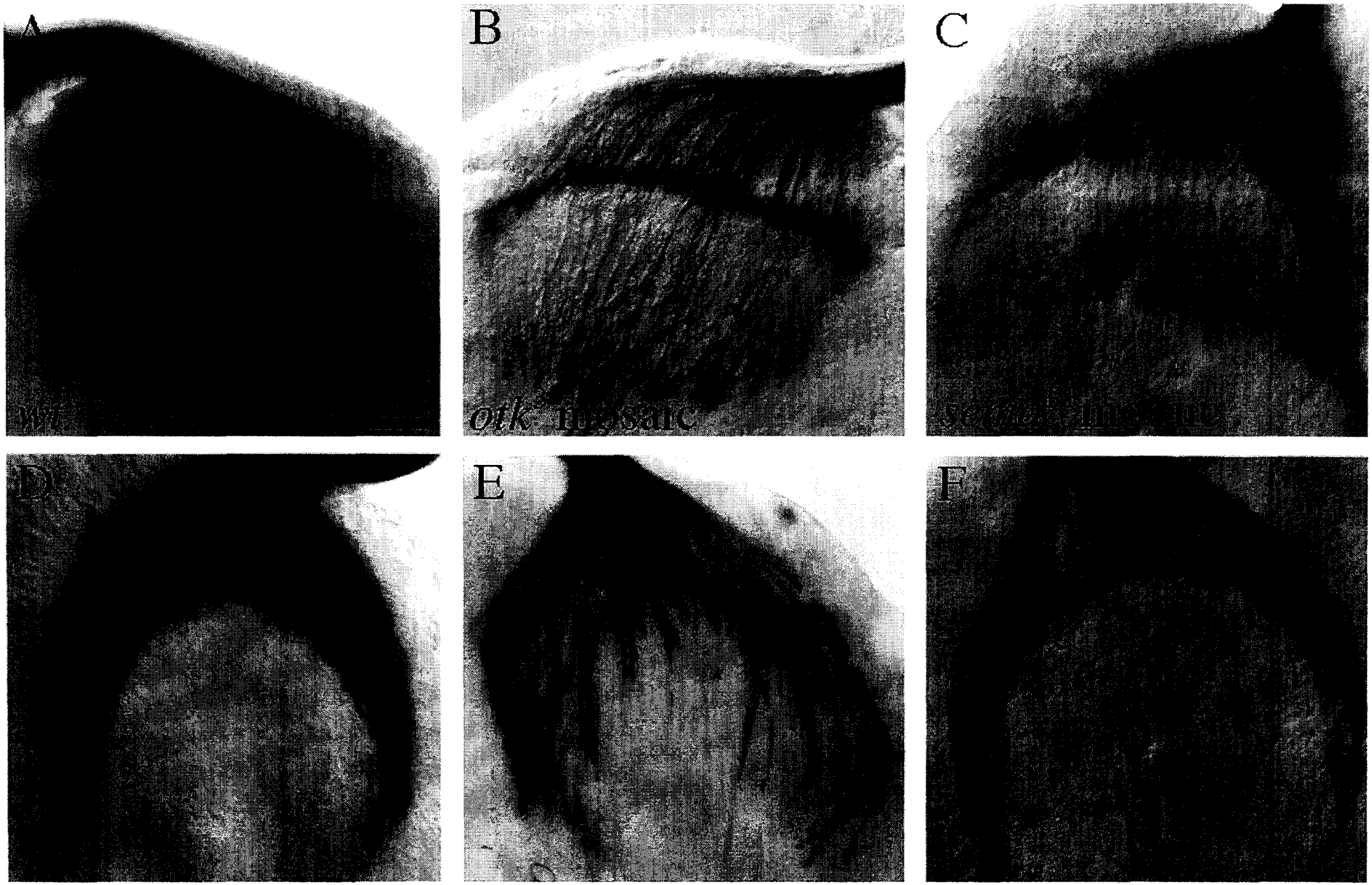

Figure 5

Figure 6. The completed pattern of R1-R6 connectivity was severely disrupted in adult *otk* mutants.

Cryostat sections of wild-type (A, C) and otk^3 eye-specific mosaic heads (B, D) were stained with MAb 24B10 to visualize all R-cell axons. In wild type (A), R7 and R8 axons projected into different layers within the medulla. Individual R7 and R8 axon terminals could be readily identified. In an otk^3 mosaic head (B), the medulla appeared to be innervated by an increased number of R-cell axons. C and D are higher magnification views of the boxed region in A and B, respectively. Whole-mount wild-type (E) and otk^3 mosaic brains (F) carrying an adult R1-R6 specific marker, Rh1-*LacZ*, were stained with anti-β-galactosidase antibody. In wild type (E), *LacZ* staining was exclusively observed in the lamina as all R1-R6 axons terminate within this layer. In an otk^3 mosaic brain (F), a large number of R1-R6 axons (arrow) connected abnormally to medulla. Scale bar, 20 μm (A, B, E, F), 5 μm (C, D).

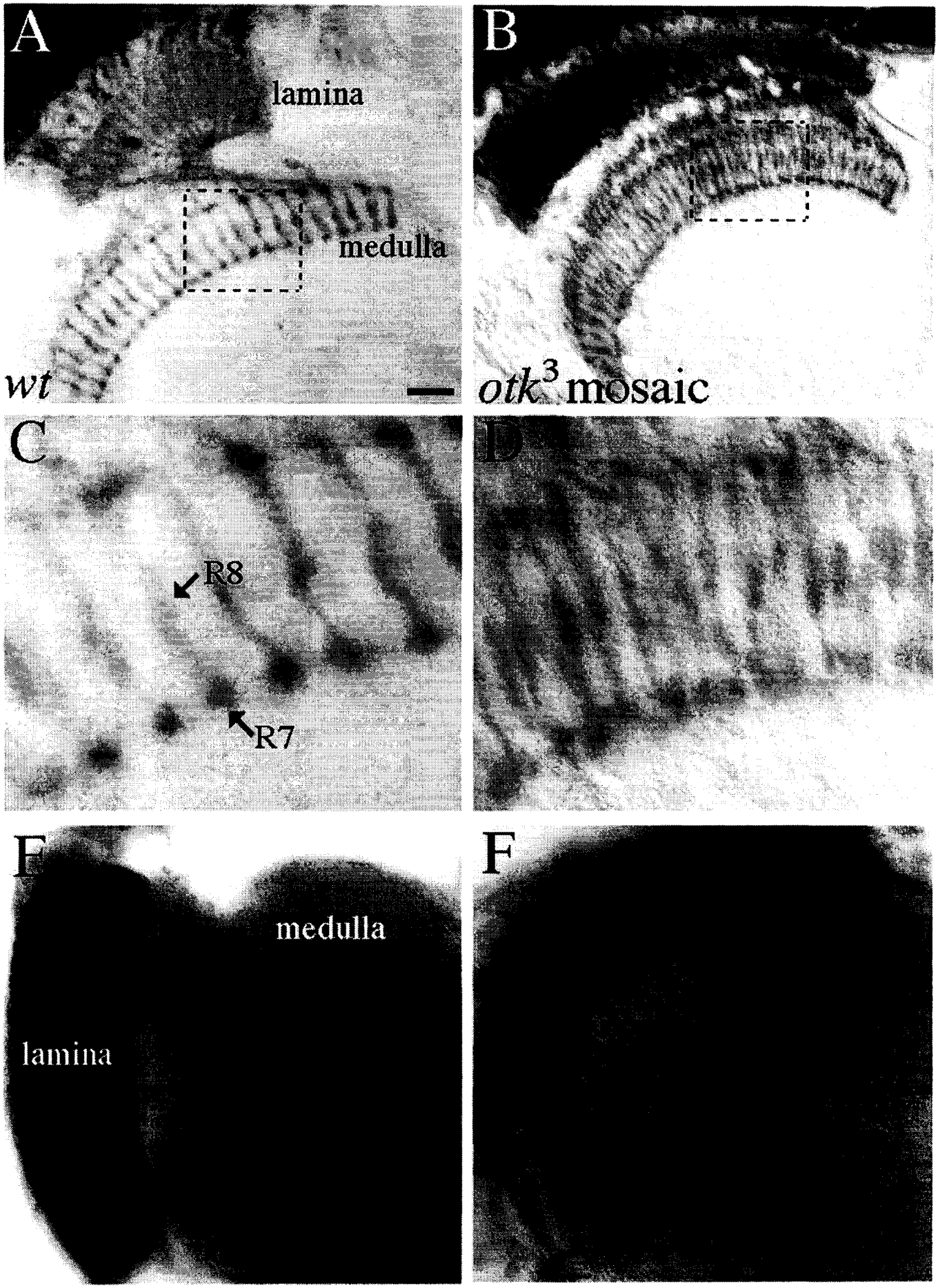

Figure 6

Figure 7. *otk* is not required for R7 targeting.

Cryostat sections of wild-type (A) and otk^3 eye-specific mosaic heads (B) were double-stained with MAb 24B10 (red) and anti-GFP antibody (green). Both wild-type and otk^3 mosaic individuals carried the adult R7 marker PANR7-GAL4 ::UAS-Synaptobrevin-GFP in which the expression of UAS-Synaptobrevin-GFP was controlled by the R7-specific driver PANR7-GAL4 (Lee et al., 2001). In wild type (A), all labeled R7 axons innervated a region that is deeper than the R8 targeting layer within the medulla. Although R7 axons in an otk^3 eye-specific mosaic head (B) appeared less organized than that in wild type (A), they still projected into the correct target region. Scale bar, 20 μm.

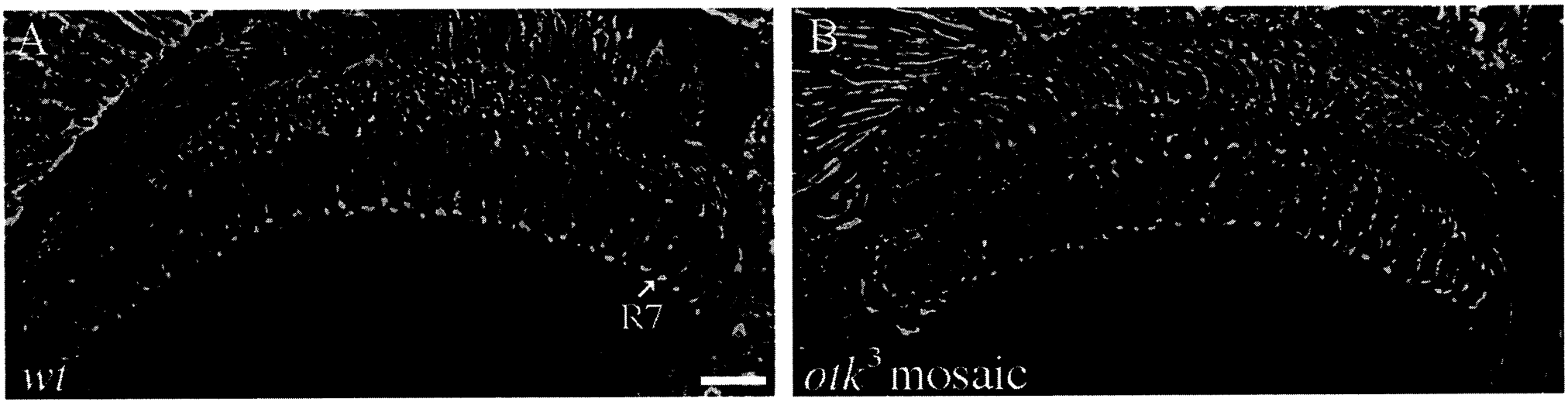

Figure 7

Figure 8. The expression of Otk in R7 did not retarget R7 growth cones to the lamina.

Otk was expressed in R7 (B) under control of the PM181-GAL4 driver. Third-instar eye-brain complexes were stained with anti-β-galactosidase. Individuals in A and B carried the PM181-GAL4 driver, an UAS-*otk* and an UAS-*lacZ* transgene. In an individual expressing Otk in R7 axons (B), like that in wild type (A), all labelled R7 axons projected correctly through the lamina (la) into the medulla (me). Scale bar, 20 μm.

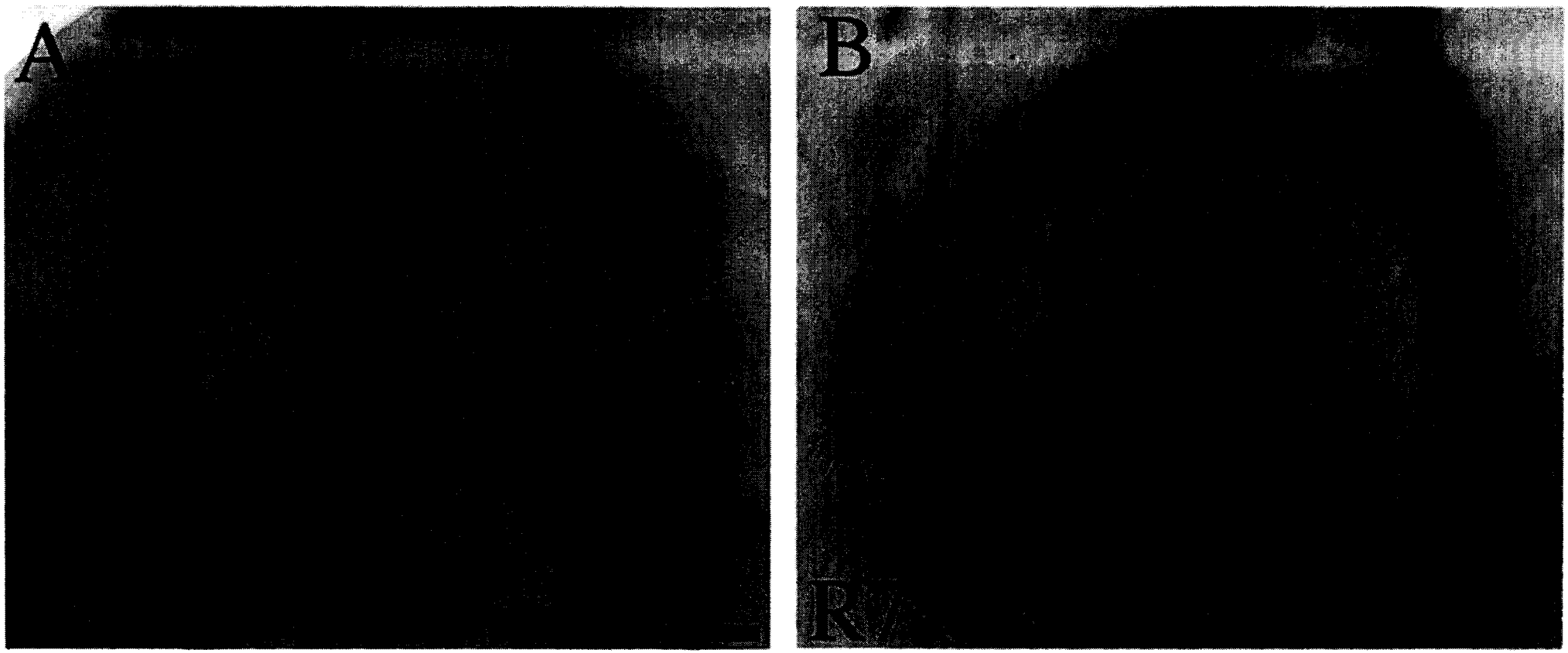

Figure 8

Discussion

In this study, we show that the receptor tyrosine kinase Otk is specifically required for lamina-specific targeting of R1-R6 growth cones. R1-R6 targeting errors in *otk* mutants were first observed at the third-instar larval stage when R-cells begin to project axons into the developing optic lobe. Many R1-R6 growth cones passed through the lamina and extended into the medulla instead. This initial R1-R6 targeting error was not corrected at a later developmental stage as many R1-R6 axons remained within the medulla in adult *otk* mutants. While *otk* is necessary for lamina-specific R1-R6 targeting, it is not required in R7 axons for establishing connections with local target cells within the medulla. The presence of Otk on R1-R6 growth cones and the specific *otk* loss-of-function phenotype support a key role for Otk in R1-R6 growth cones to specify their lamina-specific targeting decision.

The role of Otk in R1-R6 growth cones appears to be different from that of PTP69D, the only other cell surface receptor that has also been shown to be required for the initial termination of R1-R6 axons within the lamina (Garrity et al., 1999; Newsome et al., 2000a). In *Ptp69D* mutants, although ~25% of ommatidia projected one or more R1-R6 axons into the medulla at larval stage (Garrity et al., 1999), only a few axon bundles (32 mistargeted R1-R6 axons or axon bundles in a total of 34 hemispheres examined) remained within the medulla at adult stage (Newsome et al., 2000a). In addition, mutations in *Ptp69D* also disrupted R7 targeting (Newsome et al., 2000a). Many R7 axons did not project into their normal M6 layer, but instead stayed with the pioneer R8 axon at the superficial M3 layer within the medulla. These observations led to the suggestion that PTP69D plays a permissive role in R1-R6 targeting: that is, PTP69D

may mediate defasciculation between R1-R6 and the pioneer R8 axon in the lamina and between R7 and R8 axon in the medulla, thus allowing them to respond to a targeting signal. While we cannot entirely exclude this possibility for the action of Otk, it appears unlikely that R1-R6 targeting error in *otk* mutants is simply caused by defects in R-cell defasciculation. Unlike that in *Ptp69D* mutants (Newsome et al., 2000a), severe R1-R6 targeting errors (one or more mistargeted R1-R6 axons in ~42% of total ommatidial axon bundles) were also observed in *otk* adult mutants, whereas R7 target selection remained normal. Moreover, although mutations in the *trio* or *pak* gene caused a severe hyper-fasciculation phenotype, they did not affect the completed pattern of R1-R6 connectivity (Hing et al., 1999; Newsome et al., 2000b). Thus, we favor the model in which Otk is actively involved in detecting a targeting signal for R1-R6 axons to select the lamina layer.

While in *otk* mutants a large number of R1-R6 axons connected abnormally to the medulla, many R1-R6 axons still select the lamina for establishing synaptic connections. One likely explanation is that the absence of Otk may be partially compensated by another receptor that also plays a role in specifying R1-R6 targeting. Partial redundancy is not uncommon for genes that regulate axon guidance. For instance, it has been shown that four neural-specific receptor tyrosine phosphatases (i.e. PTP10D, LAR, PTP69D and PTP99A) are partially redundant with each other in regulating axon guidance in the fly embryo (Sun et al., 2001). In mammals, recent studies demonstrate that the floor-plate-derived morphogen sonic hedgehog cooperates with netrin to guide commissural axons toward the ventral midline in the developing spinal cord (Charron et al., 2003).

Previous studies show that mutations in the *brakeless (bks)* gene caused a more severe R1-R6 targeting phenotype (Rao et al., 2000; Senti et al., 2000). Most, if not all, R1-R6 axons in *bks* mutants projected aberrantly into the medulla. The *bks* gene encodes a nuclear protein expressed in all R-cells (Rao et al., 2000; Senti et al., 2000). Later studies by Banerjee and colleagues further indicate that Bks functions in R-cell growth-cone targeting by repressing the expression of another nuclear protein Runt in R2 and R5 cells (Kaminker et al., 2002). These studies thus raise the interesting possibility that Bks and Runt are components of a gene expression regulatory pathway, which controls the expression of specific cell surface receptors on R1-R6 growth cones for detecting a stop signal from the target region. To examine if the expression of Otk in R1-R6 cells is dependent on Bks, we examined the level of the Otk protein in *bks* mutants. However, no alteration in the expression level of Otk was detected (data not shown), arguing against that Otk is a downstream target of the Bks pathway.

Although *otk* is necessary for lamina-specific targeting of R1-R6 axons, its expression in R7 axons was not sufficient to target R7 axons to the lamina. There are several possible explanations for this result. Otk may need to collaborate with another cell surface protein that is present on R1-6 but not R7 growth cones to mediate the lamina-specific targeting decision, and thus act as a component of a receptor complex. This situation may be similar to that of the Nogo receptor complex that is involved in inhibiting neurite outgrowth in mammals (Wang et al., 2002). Upon ligand binding, the Nogo receptor initiates an inhibitory response only in the presence of p75, another cell surface receptor. Alternatively, the signaling components that function downstream of Otk in R1-6 growth cones may not be present in R7 growth cones or the presence of

some inhibitory mechanisms within R7 growth cones prevents them from responding to an Otk-mediated lamina-targeting signal. The possibility that Otk plays a permissive but not instructive role in R1-R6 growth-cone targeting cannot be excluded either.

Previous studies demonstrated that Otk forms a receptor complex with Plexin A that functions downstream of Sema-1a during motor axon guidance in the fly embryo (Winberg et al., 2001). In the fly adult visual system, however, the *sema-1a* phenotype appears quite different from that of *otk* as R1-R6 targeting pattern remained largely normal in *sema-1a* mutants (see Fig. 5). The simplest interpretation of this data is that *otk* functions in a different pathway in R1-R6 growth cones for specifying lamina-specific targeting decision. An alternative explanation is that Sema-1a may function redundantly with other proteins, for instance, other members of the Semaphorin protein family, to regulate the function of Otk during R1-R6 targeting. Our present data does not allow us to distinguish among these possibilities.

Otk belongs to the evolutionarily conserved CCK-4 family of "dead" receptor tyrosine kinases (Kroiher et al., 2001). Members of this family carry alterations in several evolutionarily conserved residues within the kinase domain that have been shown to be essential for the activity of most (if not all) active tyrosine kinases. Indeed, several of them have been shown to be inactive kinases by biochemical analysis (Miller and Steele, 2000). How does a defective receptor tyrosine kinase like Otk transduce targeting signals for specifying layer-specific R-cell connectivity? One possibility is that Otk associates with an unknown active tyrosine kinase, which induces tyrosine phosphorylation on Otk upon ligand binding. One precedent for this is the dead kinase ErbB3, a member of the vertebrate EGFR family. Although the kinase activity of ErbB3 is greatly impaired, it can

transduce mitogenic signals by forming a heterodimer receptor complex with another EGFR family member (e.g. ErbB2) carrying an active kinase domain (Alimandi et al., 1995; Kim et al., 1998; Sliwkowski et al., 1994). ErbB2 then induces tyrosine phosphorylation in the cytoplasmic domain of ErbB3, which serve as docking sites for downstream signaling proteins. Interestingly, it has been shown that Otk is phosphorylated on tyrosine residues in both fly and mammalian cultured cells (Pulido et al., 1992; Winberg et al., 2001). It is highly possible that in response to a targeting signal these phosphorylation sites recruit downstream signaling proteins, which then transduce the signal into the termination of R1-R6 growth cones within the lamina. In this context, it is notable that the intracellular signaling protein Dreadlocks (Dock), a SH2/SH3 adapter protein, also plays a role in lamina-specific targeting of R1-R6 axons (Garrity et al., 1996). Dock contains a single SH2 domain that can bind to specific phosphorylated tyrosine residues on activated proteins. Our previous studies suggest that a Dock-mediated signal activates the Ste20-like kinase Msn, which in turn phosphorylates the cytoskeletal regulator Bif leading to the termination of R1-R6 growth cones in the lamina (Ruan et al., 2002; Ruan et al., 1999). We have performed experiments to investigate the potential interaction between Otk and Dock during R1-R6 targeting. However, we did not observe any genetic interaction between them (data not shown). Moreover, quantification of the R1-R6 targeting phenotype in adults shows that the phenotype in *dock* mutants was less severe than that in *otk* mutants (data not shown). While this data appears inconsistent with the notion that Otk and Dock function in the same pathway, it does not exclude the possibility that Dock cooperates with another SH2-containing protein to transduce the

signal from the activation of Otk to downstream effectors for lamina-specific targeting of R1-R6 axons. Further studies will be necessary to critically address this matter.

In summary, our present study demonstrates an essential role for Otk in specifying R-cell connectivity. We propose that Otk is involved in recognizing a layer-specific signal for R1-R6 axons to select the lamina for synaptic connections. Further biochemical, molecular, and genetic dissection of the Otk pathway will help to understand the action of Otk in R-cell growth cones and shed light on the general mechanisms controlling the establishment of layer-specific neuronal connectivity in the nervous system.

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Chapter 3

In chapter 2, we provided evidence that Otk function in the lamina-specific targeting of R1-R6 axons is independent of Sema1a signaling. Sema1a, demonstrated as being a ligand necessary for the defasciculation of motor axons at specific choice points in *Drosophila*, belongs to a family of molecules that have more recently been described as also acting as attractive guidance cues. In this chapter, we describe an additional role for Sema-signaling: we find that Sema1a is required for the promotion of R-cell axonal fasciculation. Additionally, we observe that Sema1a-mediated axonal adhesion requires Sema1a to act as a receptor.

Semaphorin-1a Functions as An Axon Guidance Receptor in the Drosophila Visual System

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Summary

The evolutionarily conserved Semaphorin family proteins, including both secreted and membrane-associated forms, are well-known axon guidance ligands that mediate repulsive responses in both invertebrates and vertebrates. Several studies indicate that Semaphorins can also function as attractive guidance ligands for certain growth cones. In this study, we show that the Drosophila Semaphorin-1a (Sema1a), a transmembrane Semaphorin, is specifically required in adult photoreceptor (R-cell) axons for the establishment of an appropriate topographic termination pattern in the optic lobe. Loss of *sema1a* causes a failure for neighboring R-cell growth cones to establish close contacts leading to specific defects in local retinotopy, while overexpression of *sema1a* induces the hyper-fasciculation of R-cell axons. The function of Sema1a in R-cell axon guidance requires its cytoplasmic domain. We propose that Sema1a functions as a receptor in regulating R-cell axon guidance in the Drosophila visual system.

Key words: Semaphorin-1a, axon guidance, axon guidance receptor, Drosophila visual system

Introduction

Neuronal growth cones respond to a variety of attractive and repulsive cues present in the surrounding environment in guiding growing axons toward their target region (Tessier-Lavigne and Goodman, 1996). These cues bind to their receptors on the surface of growth cones and subsequently triggers intracellular signaling events leading to directed growth-cone movement. Among several large families of evolutionarily conserved axon guidance ligands, the Semaphorin family proteins are best known for their role in inducing repulsive responses in both invertebrates and vertebrates (Pasterkamp and Kolodkin, 2003; Tamagnone and Comoglio, 2000).

The Semaphorin family proteins can be divided into eight sub-classes (Pasterkamp and Kolodkin, 2003; Tamagnone and Comoglio, 2000), all of which are characterized by the presence of a highly conserved ~500 amino acid Sema domain. Two sub-classes of Semaphorins have been identified in invertebrates including the membrane-bound Sema1 and the secreted Sema2 classes (Pasterkamp and Kolodkin, 2003; Tamagnone and Comoglio, 2000). While Semaphorin proteins have generally been described as repulsive guidance cues, several studies indicate that some Semaphorins can also induce attractive responses (Dalpe et al., 2005; Polleux et al., 2000; Wong et al., 1999). The action of Semaphorins is mediated by two families of growth-cone receptors, plexins and neuropilins (Fujisawa and Kitsukawa, 1998). Two cell surface proteins, the receptor tyrosine kinase Off-track (Otk) in Drosophila (Winberg et al., 2001) and the cell adhesion molecule L1 in mammals (Castellani et al., 2000), have been shown to function as part of the receptor complex for Semaphorins in axon guidance.

The Drosophila transmembrane Sema1a has been shown previously to function as a repulsive guidance ligand in mediating the defasciculation of motor axon bundles at specific choice points in the fly embryo (Yu et al., 1998). Sema1a binds to its receptor Plexin A (PlexA) (Winberg et al., 1998), which in turn triggers downstream signaling events involving the receptor tyrosine kinase Otk (Winberg et al., 2001), the evolutionarily conserved flavoprotein monooxygenase MICAL (Terman et al., 2002), and the A kinase anchoring protein Nervy (Terman and Kolodkin, 2004), leading to a repulsive growth-cone response. In addition to its role in axon guidance, Sema1a has also been shown to be involved in synaptic formation (Godenschwege et al., 2002). That overexpression of wild-type Sema1a, but not a truncated Sema1a mutant protein lacking the cytoplasmic domain, caused a gain-of-function phenotype (Godenschwege et al., 2002), raises the interesting possibility that Sema1a functions as a receptor in synaptic formation. However, since the cytoplasmic domain deletion Sema1a mutant still rescued the *sema1a* loss-of-function synaptic formation phenotype (Godenschwege et al., 2002), it remains unknown if endogenous Sema1a can truly function as a receptor in the nervous system.

In this study, we show that Sema1a plays a specific role in the guidance of photoreceptor (R-cell) axons in the adult Drosophila visual system. The adult visual system is composed of the compound eye and the optic lobe. The compound eye consists of approximately 800 repeating units called ommatidia, each contains eight different R-cells. R-cells differentiate in a sequential fashion beginning with R8, followed by R2 and R5, R3 and R4, R1 and R6, and finally R7 (Tomlinson and Ready, 1987). During the third-instar larval stage R-cells extend their axons from the eye-imaginal disc through the

optic stalk into the optic lobe of the brain (Meinertzhagen and Hanson, 1993; Clandinin and Zipursky, 2002; Tayler and Garrity, 2003). After reaching the lamina, R1-R6 growth cones terminate at appropriate topographic locations, where they form close contacts with neighboring growth cones. R7 and R8 growth cones pass through the lamina and elaborate a precise topographic map in the medulla.

Our previous study implicates a role for Otk, a component of the PlexA receptor complex for Sema1a in mediating the defasciculation of embryonic motoneuron axons (Winberg et al., 2001), in layer-specific targeting of a subset of R-cell axons (i.e. R1-R6) in the adult visual system (Cafferty et al., 2004). The function of Otk in R-cell axons, however, appears to be independent of Sema1a as the R-cell projection pattern in *sema1a* null mutants was different from that in *otk* mutants (Cafferty et al., 2004). In this study, we show that Sema1a is expressed in R-cell axons and growth cones. Loss-of-function and gain-of-function analyses suggest that *sema1a* plays an attractive role in R-cell axons for the establishment of appropriate topographic projections in the optic lobe. The cytoplasmic domain of Sema1a is absolutely required for its function in R-cell axon guidance. These results are consistent with a role for Sema1a to act as a guidance receptor to regulate R-cell growth-cone interaction.

Materials and methods

Genetics

Sema1a and Fas II were overexpressed in R-cell axons by crossing *UAS-sema1a* and *UAS-Fas II* flies with the *GMR-GAL4* driver line, respectively. Transgene rescue was performed by crossing *elav-GAL4 (C155); Df(2)N22-5/Bc* flies with *UAS-sema1a*, $sema^{P1}/Bc$. The R-cell projection pattern in *elav-GAL4 (C155)/+; UAS-sema1a*, $sema^{P1}$/ *Df(2)N22-5* was compared to that in *UAS-sema1a*, $sema^{P1}$/ *Df(2)N22-5* or *elav-GAL4 (C155)/+; UAS-* $sema1a^{\Delta cyt}$, $sema^{P1}$/ *Df(2)N22-5*. To generate single *sema1a* mutant R-cell axons, *hsFLP, UAS-mCD8::GFP, elav-GAL4 (C155);* $sema^{P1}$, FRT40A/+ flies were crossed with *Tub-GAL80*, FRT40A flies. The progeny were heat-shocked at 37 °C for 1 hr at larval stage to induce mitotic recombination. R7 projections in *sema1a* mutants were examined by crossing the adult R7 marker PANR7-GAL4 ::UAS-Synaptobrevin-GFP into *sema1a* mosaics as described (Lee et al., 2001). To completely remove the *MICAL* gene in R-cells overexpressing Sema1a, genetic crosses were performed to generate the larvae with the genotype: *eyFLP; GMR-GAL4, UAS-sema1a/+; FRT82B,* $Df(3R)swp2^{MICAL}/FRT82B$, $w^{+}M(3)RpS3^{2}$.

Histology and immunohistochemistry

Plastic sectioning of adult eyes was performed as described (Garrity et al., 1996). Cryostat sections of adult mosaic heads were stained with mAb 24B10 or anti-GFP antibody as described (Garrity et al., 1996). Eye-brain complexes from third-instar larvae were dissected and stained as described (Ruan et al., 1999). MAb 24B10 and anti-GFP antibodies were used at 1:200 and 1:1000 dilutions, respectively. The secondary

antibodies (Jackson Immunochemicals) were used at 1:200 dilution. Epifluorescent images were captured using a high-resolution fluorescence imaging system (Canberra Packard) and analyzed by 2D Deconvolution using MetaMorph imaging software (Universal Imaging, Brandywine, PA). The severity of the R-cell hyper-fasciculation phenotype was quantified by counting the number of R-cell axon bundles that were located between lamina and medulla.

Results

Sema1a is expressed in R-cell axons and growth cones

To determine if *sema1a* plays a role in R-cell axon guidance, we examined if Sema1a is expressed in R-cell axons at the third-instar larval stage when the adult R-cell-to-optic-lobe connection pattern begins to form. The distribution of Semala in the developing visual system was examined using an affinity-purified anti-Sema1a antibody. At the third-instar larval stage, precursor R-cells in the eye-imaginal disc begin to differentiate into R-cells that project axons through the optic stalk into the optic lobe. The R-cell projection pattern at this stage can be visualized by staining using MAb 24B10 (Fig. 1A), which recognizes the R-cell-specific cell adhesion molecule Chaoptin (Van Vactor et al., 1988). Strong Sema1a staining was observed in R-cell bodies and their axons in the eye disc, the optic stalk, and the optic lobe (Fig. 1B, C). Within the lamina, the staining was present in R-cell axons as well as R1-R6 growth cones in the lamina plexus. Strong staining was also observed throughout the medulla neuropil comprising of R7 and R8 axons as well as non R-cell axons. We conclude that Sema1a is present in R-cell axons and their growth cones.

sema1a is required in R-cells for the establishment of an appropriate topographic termination pattern in the optic lobe

To determine the role of Sema1a in R-cell axon guidance, we performed a detailed phenotype analysis of *sema1a* loss-of-function mutants. In wild type (Fig. 2A), the differentiating R-cells send out axons toward the posterior end of the eye disc where they converge and subsequently enter the optic stalk. After exiting the optic stalk, R-cell

axons fan out to migrate over the superficial lamina. Upon reaching their appropriate topographic locations in between two layers of lamina glial cells, R1-R6 growth cones stop extension, expand significantly in size, and form close contacts with neighboring growth cones, resulting in the establishment of a continuous and dense terminal layer in the lamina (Fig. 2A). Whereas R7 and R8 growth cones pass through the lamina into the medulla, where they also expand in size and elaborate a precise topographic termination pattern (Fig. 2A).

In homozygous *sema1a*^{P1} mutant larvae (Fig. 2B, C), the initial outgrowth of R-cell axons appeared normal. Mutant R-cell axons migrated correctly from the eye disc into the optic stalk, which was morphologically indistinguishable from that in wild type. After R-cell axons exited from the optic stalk en route to their termination region, however, severe defects were observed (Fig. 2B, C). R1-R6 growth cones failed to pack into a dense termination layer in all mutant hemispheres examined (n=11). Instead, they scattered around the lamina terminal field and appeared to be unable to establish a close association in the target region. Some R1-R6 axons did not stop at their appropriate topographic termination region, and instead migrated laterally into incorrect locations in the lamina (Fig. 2B). The array of R7 and R8 growth cones in the medulla also appeared to be slightly disorganized. Similar expressivity of phenotype were observed in *sema1a*^{P1} hemizygotes (n=30 hemispheres, Fig. 2D, E), consistent with the null nature of this allele (Yu et al., 1998). To determine if the above defects reflect a role for *sema1a* in R-cells, we examined the projection pattern of *sema1a* mutant R-cell axons in an otherwise heterozygous or wild-type target region by using genetic mosaic analysis. Large clones of homozygous *sema1a*^{P1} mutant eye tissues were generated by eye-specific mitotic

recombination using the *eyFLP/FRT* system (Newsome et al., 2000). A similar phenotype was observed in all *sema*^{P1} mosaic individuals examined (n = 20 hemispheres, Fig. 2F), indicating that *sema1a* is required in R-cells for the guidance of R-cell axons.

While severe defects in local retinotopy were observed in *sema1a* mutants, the lamina-versus-medulla target selection appeared largely normal. The majority of R2-R5 axons, a subset of R1-R6 axons labeled by the *ro-τ-lacZ* marker, still stop within the lamina layer in *sema1a* mutants (n=15, compare Fig. 2L to 2K). Consistent with the phenotype observed with MAb 24B10 staining (Fig. 2B-F), we found that the organization of R2-R5 axons in the lamina plexus was disrupted in *sema1a* mutants (Fig. 2L).

sema1a is required autonomously in R1-R6 axons

The above defects observed in *sema1a* mutants might reflect an autonomous role for Sema1a in a single R-cell axon. Alternatively, Sema1a might be required for neighboring axon projection in a non-cell-autonomous manner. To distinguish between these possibilities, we examined the projection of single mutant axons using the mosaic analysis with a repressible marker (MARCM) method (Lee and Luo, 1999).

In wild type, R1-R6 axons terminate in between two layers of glial cells (i.e. epithelial and marginal glia) in the lamina in a highly organized topographic pattern (Fig. 3A). When large patches (>90% eye tissues) of *sema1a* mutant R-cells were generated using eye-specific mitotic recombination, although the organization of glial cells in the lamina remained normal, severe defects in the R1-R6 growth-cone termination pattern were observed (Fig. 3B). R1-R6 growth cones were observed in a much wider region

between lamina epithelial glial cells and medulla glial cells as many of them passed over the marginal glial layer.

To determine the projection pattern of single mutant R-cell axons, we generated single *sema1a* mutant mosaics in the eye by expressing the FLP recombinase under the control of heat-shock-inducible promoter at larval stage. In control (wild-type single mosaics), most labeled single R1-R6 axons (77%, n=96 single axons in 15 hemispheres) terminated normally in the region between epithelial and marginal glial cells (Fig. 3C). In contrast, most labeled single *sema1a* mutant R1-R6 axons (71%, n=128 single axons in 11 hemispheres) displayed abnormal projection pattern (Fig. 3D and 3E). Many of them terminated in between lamina marginal glia and medulla glia (Fig. 3D). Some mutant axons did not terminate at appropriate topographic locations and instead extend laterally within the lamina (Fig. 3E). These results indicate an autonomous role for Sema1a in R1-R6 axons.

R-cell differentiation and retinal patterning remain normal in *sema1a* mutants

To determine if the above R-cell axon guidance defects were due to abnormal eye development, we examined R-cell differentiation and patterning in *sema1a* mutant eye disc and adult mosaic eye. No defects in either the differentiation or the organization of R-cell clusters were observed in third-instar eye discs in *sema1a* mutants (compare Fig. 4B to A). Plastic sectioning of adult *sema1a* mosaic eye did not reveal any defect in either the number or the organization of R-cells within each mutant ommatidium (0 out of 996 mutant ommatidia examined, compare Fig. 4D to C). The gross organization of mutant ommatidia in each clone also appeared normal. These data exclude the possibility

that the R-cell axon guidance phenotype in *sema1a* mutants is secondary to abnormal R-cell development in the eye.

Overexpression of *sema1a* induced the hyper-fasciculation of R-cell axons

That R1-R6 growth cones failed to establish a highly condensed R1-R6 terminal layer in *sema1a* mutants is inconsistent with a repulsive role for Sema1a, which has been shown previously to be necessary for the defasciculation of embryonic motor axon bundles at specific choice points (Yu et al., 1998). Instead, the above phenotype in the adult visual system would be explained more easily with a model in which Sema1a is involved in mediating an attractive interaction. To further address this possibility, we examined if overexpression of Sema1a in R-cell axons would cause an opposite phenotype, for instance, an increase in the association of R-cell axons.

Sema1a was overexpressed in R-cell axons by using the eye-specific *GMR-GAL4* driver. When the *GMR-GAL4* driver was used to overexpress Fasciclin II (Fas II), a well-known homophilic cell adhesion molecule required for axonal fasciculation (Lin et al., 1994), we observed an axonal hyper-fasciculation phenotype (Fig. 5B, Table 1). If Sema1a, like Fas II, plays an attractive role in R-cell axons, one would predict that overexpression of Sema1a should produce a similar hyper-fasciculation phenotype. Overexpression of Sema1a was confirmed by staining R-cells with an anti-Sema1a antibody (data not shown). Indeed, we found that overexpression of Sema1a caused a hyper-fasciculation phenotype similar to that in larvae overexpressing Fas II (compare Fig. 5C to B, Table 1). Thicker axon bundles were formed in both lamina and medulla in all hemispheres examined (n=50), co-incident with the presence of large clumps of

terminals in the lamina plexus. This phenotype is dosage-dependant as an increase in the dosage of the *sema1a* transgene dramatically enhanced the phenotype (100%, n = 35, Fig. 5D, Table 1). When both Sema1a and Fas II were overexpressed in R-cell axons, the hyper-fasciculation phenotype was dramatically enhanced (Fig. 5E, Table 1). This result is in marked contrast to the previous observation that *sema1a* counters the attractive action of Fas II in embryonic motor axons (Yu et al., 2000), and is consistent with an attractive role for Sema1a in R-cell axon guidance.

Sema1a has been shown to bind to its receptor PlexA to mediate repulsive interactions between motor axons (Winberg et al., 1998). To determine if the above Sema1a-induced R-cell hyper-fasciculation phenotype is also dependent on the activation of the PlexA signaling pathway, we examined the potential epistatic interaction between *sema1a* and genes in the *PlexA* pathway. We found that the complete loss of the *MICAL* gene, which functions downstream of *PlexA* in both invertebrates and vertebrates (Terman et al., 2002), did not modify the *sema1a* overexpression phenotype (n=19, Fig. 5F, Table 1). This result suggests that the attractive function of Sema1a in R-cell axons is independent of the classical PlexA signaling pathway.

The cytoplasmic domain of Sema1a is indispensable for its action in R-cell axon guidance

The above loss-of-function and gain-of-function analyses support a specific role for Sema1a in R-cell axons. Sema1a may function as a ligand that activates its receptor on neighbouring R-cell growth cones to promote their association. Alternatively, since Sema1a contains a cytoplasmic domain, it may function as a guidance receptor to mediate

the interactions between R-cell axons, which is supported by that *sema1a* is required autonomously in R1-R6 axons (Fig. 3). To further determine the role of Sema1a in R-cell axons, we examined if the cytoplasmic domain of Sema1a is essential for its action in R-cell axon guidance.

Firstly, we tested if deleting the cytoplasmic domain of Sema1a affects its ability to induce the hyper-fasciculation phenotype. The *sema1a*$^{\Delta cyt}$ transgene encoding a membrane-associated Semala mutant protein in which the carboxyl-terminal amino-acid sequence 695-899 of the cytoplasmic domain (aa 680-899) is deleted (Godenschwege et al., 2002), was overexpressed in R-cell axons. Surprisingly, we found that overexpression of *sema1a*$^{\Delta cyt}$ was unable to induce the hyper-fasciculation of R-cell axons (0 out of 23 hemispheres, Fig. 6B, Table 1), but instead caused a phenotype indistinguishable from that in *sema1a* loss-of-function mutants (~80%, n=23, compare Fig. 6B to Fig. 2B-F). This result indicates that *sema1a*$^{\Delta cyt}$ acts as a dominant-negative form to interfere with the function of endogenous Sema1a.

We then tested if the cytoplasmic domain is required for rescuing the *sema1a* loss-of-function phenotype. Neuronal-specific expression of wild-type *sema1a* rescued the phenotype (14 out of 22 mutant hemispheres, Fig. 6C). In contrast, all mutant hemispheres expressing *sema1a*$^{\Delta cyt}$ still displayed the *sema1a* loss-of-function phenotype (n=15, compare Fig. 6D to Fig. 2B-J), indicating that the cytoplasmic domain is essential for the function of Sema1a in R-cell axons. This data, taken together with that overexpression of *sema1a*$^{\Delta cyt}$ in wild-type larvae caused a *sema1a*-like loss-of-function phenotype (see above), suggests strongly that Sema1a functions as a receptor in R-cell axon guidance.

Figures

Figure 1. Sema1a is present in R-cell axons and growth cones in the developing visual system.

(A and B) Wild-type third-instar larval eye-brain complexes were double stained with MAb 24B10 (green), which recognizes all R-cell axons, and anti-Sema1a (red). The merge is shown in C. Sema1a staining is present in R-cell bodies in the eye disc and their axonal trajectories from the eye disc through the optic stalk into the developing optic lobe. The lamina plexus consisting mainly of R1-R6 growth cones is strongly stained. The uniformly staining pattern in the medulla indicates that Sema1a is also present in non-R-cell axons. (D and E) *sema1a*^{P1} mosaic eye-brain complexes in which almost entire eye tissues were homozygous *sema1a*^{P1} mutant cells, were double stained with MAb24B10 (D) and anti-Sema1a (E). The merge is shown in F. The staining is absent in R-cell bodies and axons, confirming the specificity of this antibody. Scale bar: 20 μm.

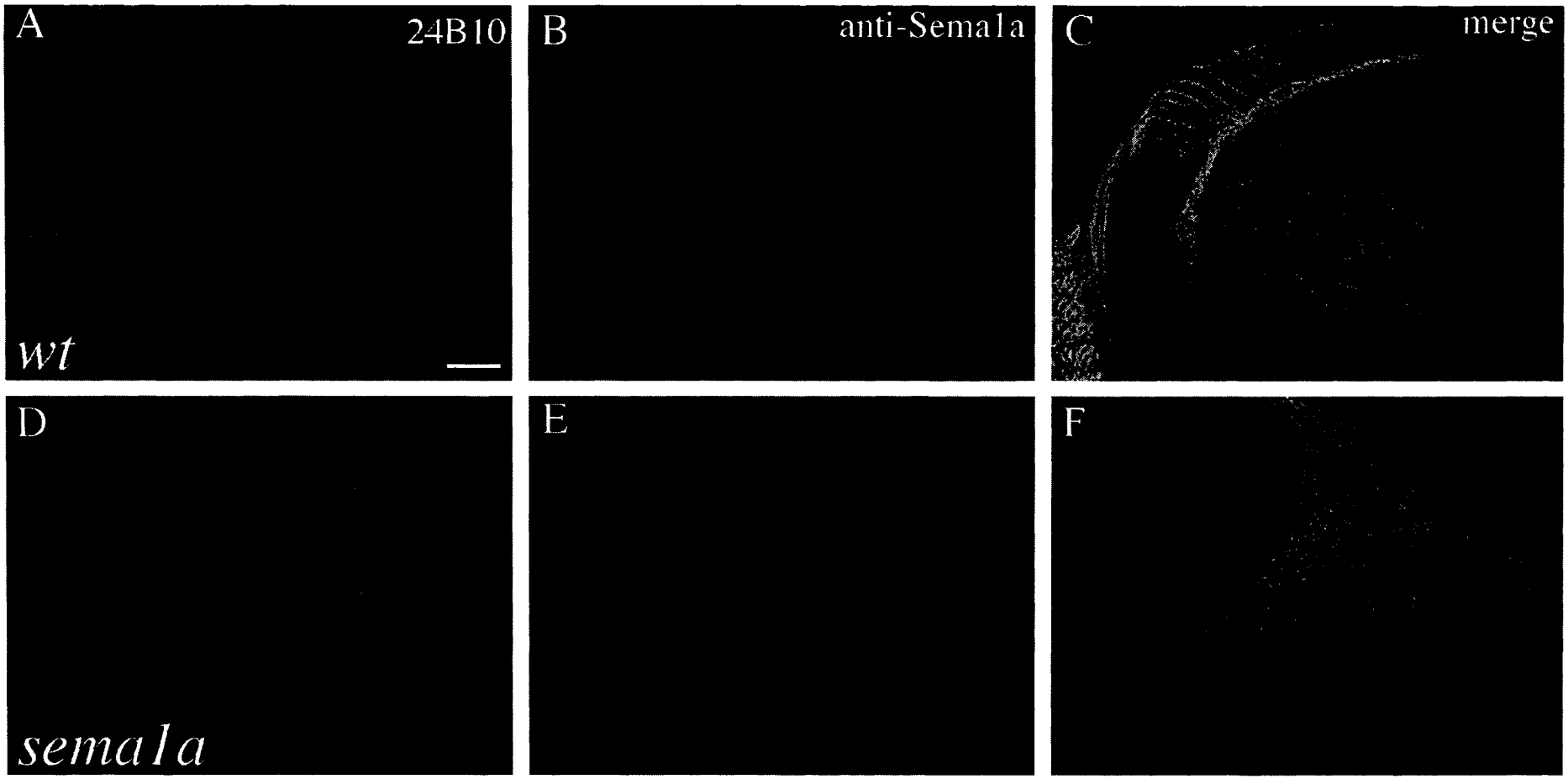

Figure 1

Figure 2. *semala* is specifically required for the establishment of an appropriate local retinotopic termination pattern.

(A-J) Third-instar larval eye-brain complexes were stained with MAb 24B10 to visualize all R-cell axons. In wild type (A), R1-R6 axons stop within the lamina. Their expanded growth cones associate closely with each other and elaborate a smooth and dense terminal layer in the lamina. R7 and R8 axons project through the lamina into the medulla, elaborating a precise topographic map. In a *sema1a*^{P1} homozygote (B, C), R1-R6 growth cones associated loosely with neighboring growth cones, leading to the appearance of a discontinous and uneven terminal field. B and C show the same individual at different focal planes. Some R1-R6 axons did not stop at their appropriate topographic destinations and instead migrated laterally at the bottom of lamina into incorrect locations (arrow in B). The array of R7 and R8 growth cones in the medulla also appeared less organized (C). Similar phenotype was observed in a *sema1a*^{P1} hemizygote (*sema1a*^{P1}*/Df(2)N22-5*) (D, E), and a *sema1a*^{P1} mosaic individual (F) in which large clones of homozygous mutant tissues were generated in an otherwise wild-type target region by using eye-specific mitotic recombination (Newsome et al., 2000). D and E show the same individual at different focal planes. G-J are enlarged views of the boxed regions in A, C, D, F, respectively. (K and L) R2-R5 axons in wild type (K) and *sema1a*^{P1} homozygous mutants (L) were labeled with the *ro-τ-lacZ* marker. In wild type (K), only a few R2-R5 axons (~5) project into the medulla. In *sema1a*^{P1} homozygotes (L), the average number of mistargeted R2-R5 axons is ~7, which is not significantly different from that in wild type (i.e. ~5). Note that some R2-R5 axons (arrow) migrated abnormally at the bottom of lamina. Scale bar: 20 μm.

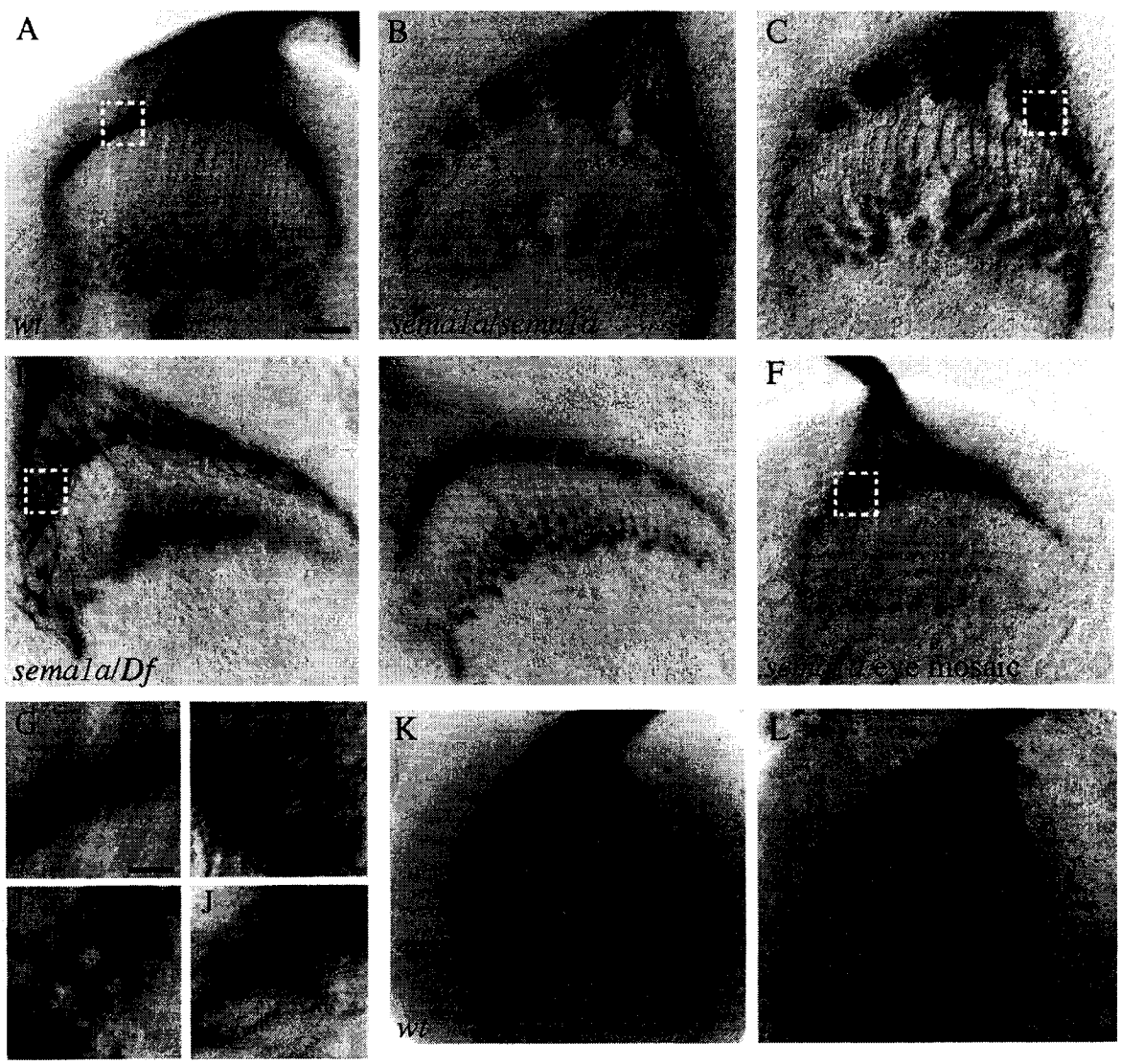

Figure 2

Figure 3. *sema1a* is required in single R1-R6 axons.

(A and B) Wild-type (A) and *sema1a*^{P1} mosaic (B) eye-brain complexes were double-stained with MAb 24B10 (green) and anti-Repo (red). The anti-Repo antibody recognizes the glial-specific nuclear protein Repo. In wild type (A), R1-R6 growth cones terminate in between epithelial (eg) and marginal (mg) layers. In large *sema1a* eye mosaic animals (B), the migration and differentiation of lamina glial cells appear normal. However, the organization of R1-R6 growth cones at the termination site was abnormal. R1-R6 growth cones were distributed in a much wider area between the lamina glial and the medulla glial cells (meg). (C-E) Single wild-type (C) or *sema1a* mutant axons (D and E) were positively labeled using the MARCM method (Lee and Luo, 1999). *sema1a* single mutant axons frequently passed over the marginal glial layer (arrow in D). Some (arrow in E) terminated at incorrect topographic locations. Scale bar: 20 μm.

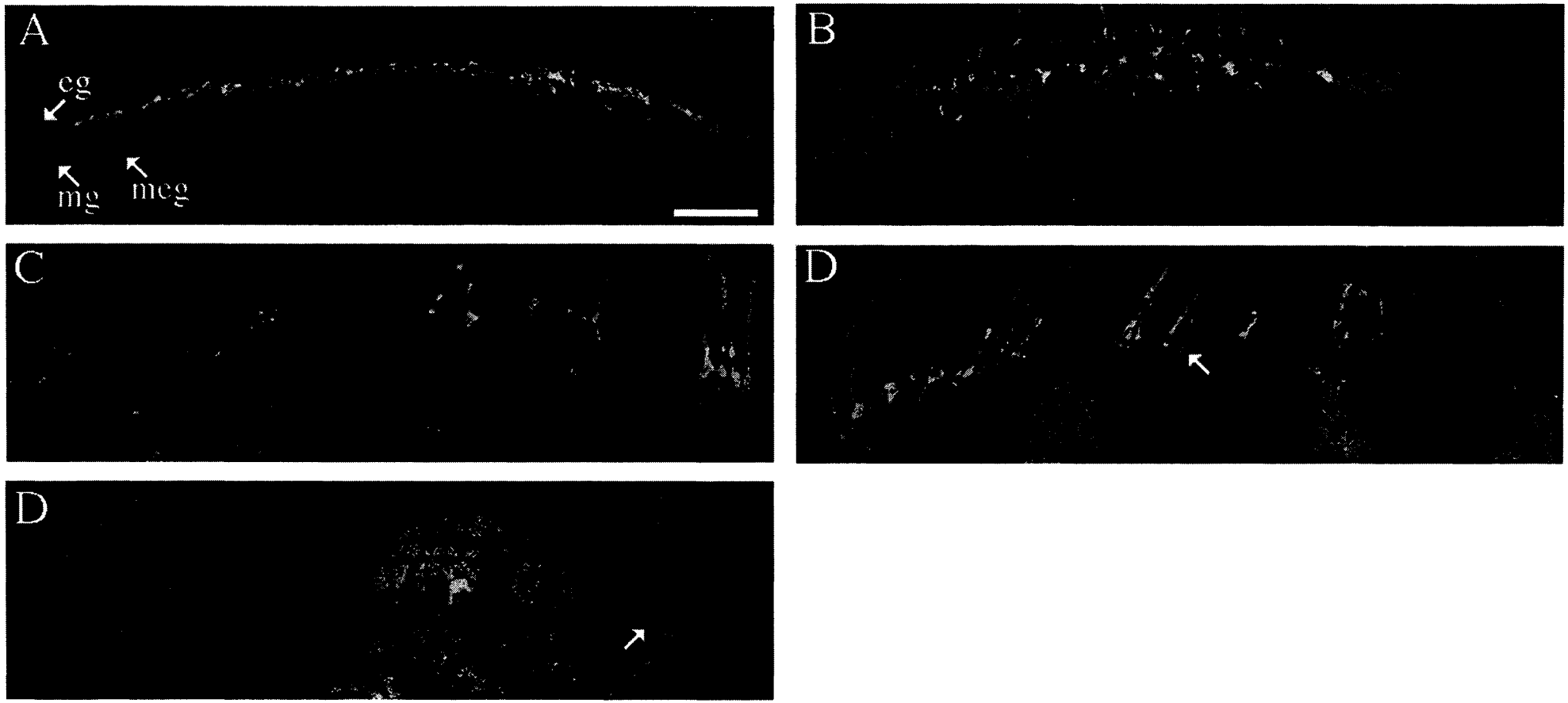

Figure 3

Figure 4. Loss of *sema1a* did not affect R-cell differentiation or retinal patterning.

(A and B) Wild-type (A) and *sema1a*^{P1} hemizygous mutant (B) eye discs were stained with MAb 24B10. The organization of differentiating R-cell clusters in *sema1a* mutants was indistinguishable from that in wild type. (C and D) Wild-type (C) and *sema1a*^{P1} mosaic (D) adult eyes were embedded in epon and sectioned. Scale bar: 20 μm in A and B; 10 μm in C and D.

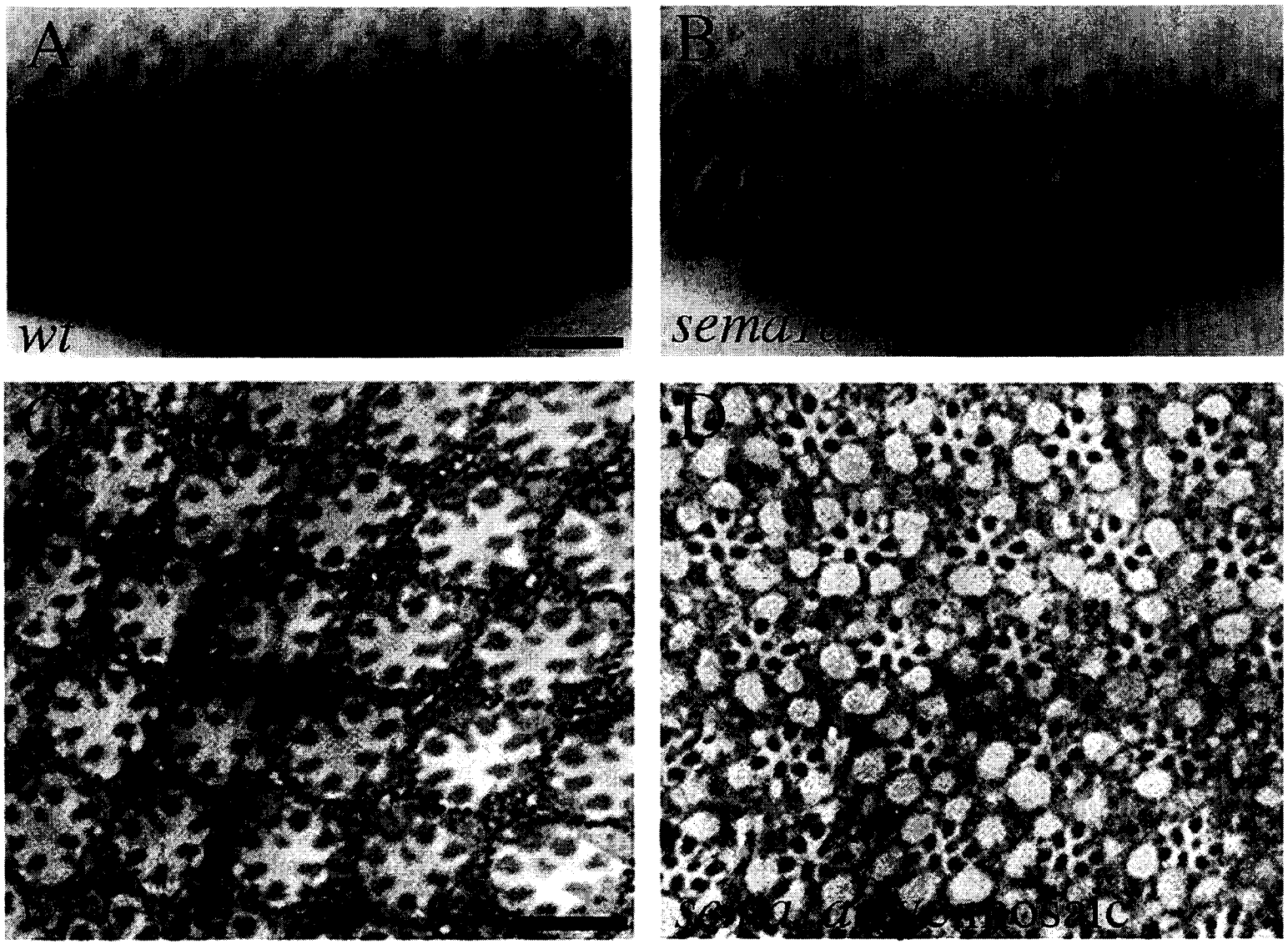

Figure 4

Figure 5. Overexpression of Sema1a induced the hyper-fasciculation of R-cell axons. (A) Wild type. (B) Overexpression of Fas II in R-cell axons induced the formation of thicker bundles. (C) Overexpression of Sema1a also caused a hyper-fasciculation phenotype. In larvae carrying two copies of the *UAS-sema1a* transgene (D), the phenotype becomes much more severe. R-cell axons form large clumps in the lamina and appeared to be unable to defasciculate and extend deeply into the medulla. In larvae carrying one copy of *UAS-Fas II* and *UAS-sema1a* (E), the phenotype is much stronger than that in larvae carrying a single copy of *UAS-Fas II* (B) or *UAS-sema1a* (C). The complete loss of the *MICAL* gene (F) did not suppress the Sema1a overexpression phenotype. Scale bar: 20 μm.

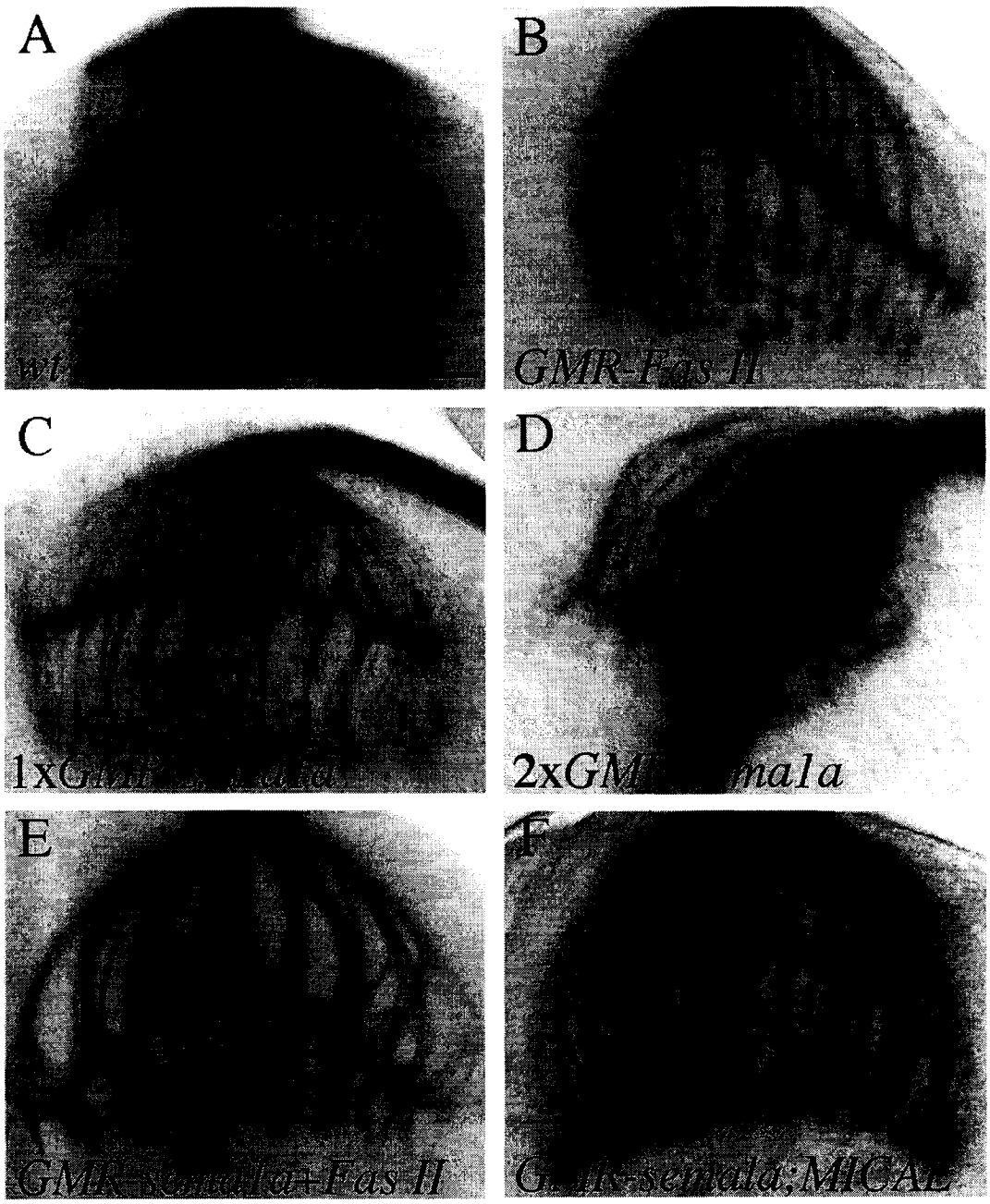

Figure 5

Figure 6. The cytoplasmic domain of Sema1a is required for its function in R-cell axon guidance.

(A) Overexpression of wild-type Sema1a caused the hyper-fasciculation of R-cell axons. (B) Overexpression of *sema1a*$^{\Delta cyt}$ did not induce the formation of thicker bundles, but instead disrupted the organization of R1-R6 growth cones in the lamina plexus, which was indistinguishable from that in *sema1a*^{P1} mutants (Fig. 2B-J). (C) Neuronal-specific expression of wild-type Sema1a in a *sema1a*^{P1} hemizygote (i.e. *sema1a*^{P1}/ *Df(2)N22-5*) restored the normal R-cell projection pattern in 14 out of 22 mutant hemispheres. Note the appearance of the continuous and dense R1-R6 terminal layer in the lamina. The remaining eight mutant hemispheres displayed the axonal hyper-fasciculation phenotype (data not shown), likely due to an above threshold expression level of the *sema1a* transgene in these individuals. (D) No rescue was observed when the *sema1a*$^{\Delta cyt}$ transgene was expressed in a *sema1a*^{P1} hemizygote. Note the discontinuous lamina plexus and the aberrant projections of some R-cell axons (arrow) at the bottom of lamina. Scale bar: 20 μm.

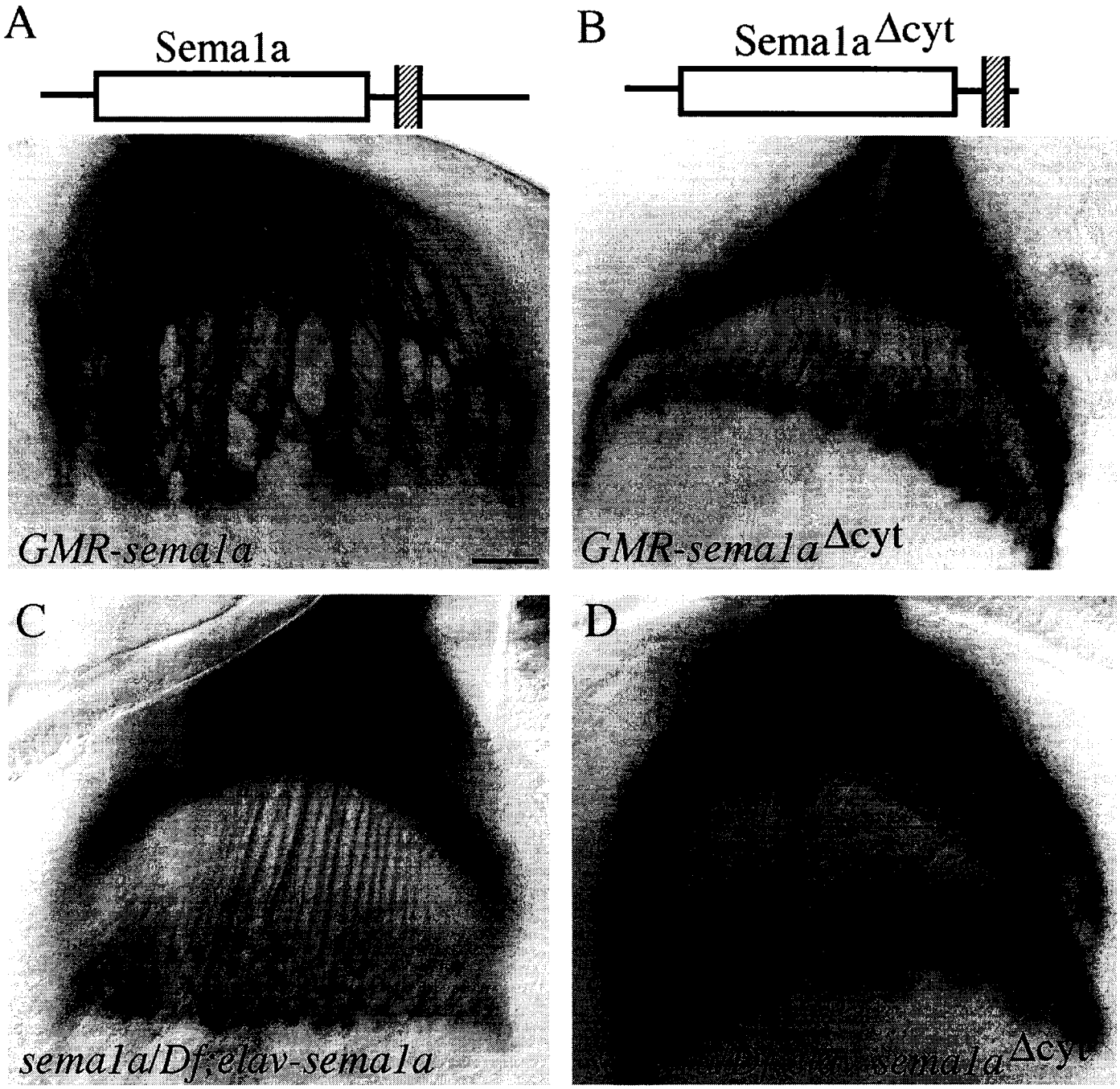
A
Sema1a
B
Sema1a$^{\Delta cyt}$
GMR-sema1a
GMR-sema1a$^{\Delta cyt}$
C
D
sema1a/Df;elav-sema1a

Figure 6

Table 1. Quantification of the axonal hyper-fasciculation phenotype in third-instar larvae

Genotype	Average number of axons or axon bundles[a]	Number of hemispheres examined
Wt	39 ± 5[b]	24
Semala^{P1}/ *semala*^{P1}	32 ± 5	13
Semala^{P1}/ *Df(2)N22-5*	35 ± 5	20
Semala^{P1} eye mosaic	38 ± 4	20
UAS-Fas II	38 ± 5	16
UAS-Sema1a	39 ± 5	13
GMR-GAL4/+	37 ± 5	13
GMR-GAL4/UAS-Fas II	20 ± 2	15
GMR-GAL4, UAS-semala/+	21 ± 3	20
2X*GMR-GAL4, UAS-semala*	7 ± 3	20
GMR-GAL4, UAS-semala/UAS-Fas II	13 ± 2	15
[c] *GMR-GAL4, UAS-semala/+; Df(3R)swp2*^{MICAL}	20 ± 5	19
GMR-GAL4/UAS-semala$^{\Delta cyt}$	37 ± 4	16

[a] The number of axons or axon bundles that separated from neighboring bundles at the region between lamina and medulla were counted. This number decreased significantly in larvae overexpressing Sema1a or Fas II due to the formation of thicker bundles.

[b] Standard deviation

[c] Sema1a was overexpressed in larvae in which almost entire eye tissues were homozygous *MICAL* mutant cells generated by eye-specific mitotic recombination.

Discussion

Semaphorin family members have been extensively studied for their role as axon guidance ligands in both invertebrates and vertebrates. Murphey and colleagues demonstrate previously that the cytoplasmic domain of Sema1a, the Drosophila transmembrane Semaphorin, is required for inducing a gain-of-function synaptic formation phenotype, raising the possibility that Sema1a functions as a receptor in synaptic formation (Godenschwege et al., 2002). Their result showing that the Sema1a mutant lacking the cytoplasmic domain still rescues the *semala* loss-of-function synaptic formation phenotype (Godenschwege et al., 2002), however, raises the question if endogenous Sema1a truly functions as a receptor in the nervous system. In this study, we provide several lines of evidence to support that Sema1a indeed functions as a receptor in the fly visual system to regulate the formation of appropriate R-cell topographic projections in the optic lobe. First, we show that *sema1a* is required autonomously in single R-cell axons. Second, unlike overexpression of wild-type Sema1a, overexpression of the membrane-bound Sema1a$^{\Delta cyt}$ mutant lacking the cytoplasmic domain is incapable of inducing a R-cell hyper-fasciculation phenotype. Third, the cytoplasmic domain of Sema1a is required for rescuing the *sema1a* loss-of-function phenotype. And finally, the Sema1a$^{\Delta cyt}$ mutant lacking the cytoplasmic domain causes a dominant-negative effect when expressed in wild-type flies.

Sema1a may function as a guidance receptor in R1-R6 axons to promote an attractive interaction between neighboring growth cones when they reach their intermediate target region at the third-instar larval stage. The local interaction between R1-R6 growth cones has been shown to provide certain guidance information for R1-R6

axons to select their appropriate synaptic partners during pupation (Clandinin and Zipursky, 2000). Similarly, we speculate that a Sema1a-dependent attractive interaction at the third-instar larval stage may allow neighboring growth cones to communicate with each other, thus facilitating the formation of an appropriate retinotopic termination pattern in the lamina. An alternative model for the action of Sema1a in the visual system is that Sema1a functions as a receptor in R1-R6 axons to detect local guidance signals present in the lamina. Our current data does not allow us to distinguish among these possibilities.

The molecular nature of the ligand for Sema1a in the visual system is unknown. Sema1a may mediate the interaction between R-cell growth cones in a homophilic fashion. Alternatively, other proteins, for instance, PlexA, the receptor for Sema1a in mediating repulsive interactions during embryonic motor axon guidance (Winberg et al., 1998), may function as a ligand for Sema1a in the visual system. Further studies are required to determine if PlexA or other proteins acts as the ligand for Semala in the visual system and understand the Sema1a-dependent downstream signaling events.

It appears highly likely that transmembrane Semaphorins in vertebrates can also function as axon guidance receptors in the nervous system. Several vertebrate Semaphorins have been shown to be able to bind via their cytoplasmic domains to intracellular signaling proteins such as EVL (Klostermann et al., 2000), PSD-95 (Inagaki et al., 2001; Ohoka et al., 2001; Schultze et al., 2001), and c-Src (Eckhardt et al., 1997). While we were preparing this manuscript, Kikutani and colleagues (Toyofuku et al., 2004) provided evidence to support the idea that the chick transmembrane Semaphorin Sema6D can function as a receptor in cell migration during embryonic development. It

will be of interest to determine if these vertebrate transmembrane Semaphorins also function as axon guidance receptors in the nervous system.

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Chapter 4

General Discussion

This chapter involves a general discussion of the significance of our findings in this thesis. First, I will address the requirement of the Otk receptor for R1-R6 axons to properly target to the lamina layer. The correct targeting of R1-R6 axons may not require Otk to possess tyrosine kinase activity and the Otk ligand in the visual system does not appear to be Sema1a. Next, I will discuss the action of Sema1a to promote R-cell growth cone attraction and the requirement of Sema1a to act as a receptor in R-cell axons. The final section of this discussion describes future work that can serve to identify the unknown ligands and downstream effectors of Otk and Sema1a in the *Drosophila* visual system

4.1 A requirement for Otk in the layer-specific targeting of R1-R6 axons

The results presented in this thesis demonstrate a requirement for Otk in the layer-specific targeting of R1-R6 axons (Chapter 2, Cafferty *et al.*, 2004). While Fmi, N-Cadherin, and LAR have been shown to be functionally required in R1-R6 axons (Lee *et al.*, 2001) and are necessary in R1-R6 axons for the formation of proper connections with normal target neurons (Clandinin *et al.*, 2001; Lee *et al.*, 2001; Lee *et al.*, 2003), *fmi*, *N-cadherin*, and *LAR* mutant R1-R6 axons all terminate normally at the lamina layer. A role for DInR in R1-R6 layer-specific targeting has not been investigated as R1-R6 axons have not been specifically labeled and examined in either third instar larva or in adult *dinr* mutants. Thus, the only identified cell-surface receptors required for the proper termination of R1-R6 axons are Otk and the protein tyrosine phosphatase PTP69D (Garrity *et al.*, 1999).

The differences in *otk* and *ptp69D* LOF phenotypes suggest different functions for each receptor in the R1-R6 targeting of axons. While 32% and 20-25% of ommatidia aberrantly projected R2-R5 axons into the medulla of *otk* and *ptp69D* mosaic third instar larva respectively, indicating a common role for Otk and Ptp69D in R1-R6 layer-specific targeting, only mutations in *ptp69D* were found to affect R7 targeting (Newsome *et al.*, 2000a). The fact that loss-of-*ptp69D* resulted in abnormal early R7 termination in the M3 layer of the medulla suggests that PTP69D functions in a permissive fashion, properly allowing R1-R6 and R7 axons to defasciculate from the R8 pioneer axon and interact with specific targeting signals. By contrast, Otk does not appear to affect R7 target selection as neither Otk overexpression in R7, nor removal of *otk* from R7 axons resulted in a change of R7 phenotype. As well, approximately 42% of ommatidia aberrantly project R1-R6 axons into the medulla of adult *otk* mutants while in adulthood only 5% of ommatidia extend axons into the medulla of *ptp69D* mutants. The lack of function in R7 axons paired with the uniquely severe adult LOF phenotype suggests that Otk acts in an instructive manner in R1-R6 axons where Otk recognizes a specific termination signal in the lamina layer. The observation that Otk is tyrosine phosphorylated in both fly lysate and mammalian cell culture (Pulido *et al.*, 1992; Winberg *et al.*, 2001) suggests that the binding of a lamina ligand to Otk may trigger a tyrosine phosphorylation event. Since Otk is a member of the Colon carcinoma kinase-4 (CCK-4) family of "dead" receptor tyrosine kinases and may not have intrinsic kinase activity (Kroiher *et al.*, 2001), the tyrosine phosphorylation event may be catalyzed by other kinases.

4.2 Otk is a member of the CCK-4 "dead" kinase family

The Otk kinase domain contains the triplet amino acid sequence ALG in place of the conserved DFG sequence known to bind magnesium that is essential for kinase activity (Kroiher *et al.*, 2001), suggesting that Otk may lack kinase activity. The requirement for an inactive kinase in the proper projection of axons is not unprecedented, as the dead receptor tyrosine kinase Derailed (Drl) is required by neurons that project into the AC in the *Drosophila* embryonic ventral nerve cord (Bonkowsky *et al.*, 1999). The Drl receptor, identified in a screen for genes expressed in restricted subsets of embryonic neurons that choose common pathways (Callahan *et al.*, 1995), is also required for proper muscle attachment site selection (Callahan *et al.*, 1996). Yoshikawa *et al.* (2001) demonstrated that a mutant form of *drl* lacking a conserved lysine required for kinase activity could both induce a GOF phenotype in the embryonic CNS and rescue the loss-of-*drl* muscle attachment phenotype, indicating that Drl function does not require intrinsic kinase activity. As autophosphorylation of dead receptor kinases is impossible, kinase-dead receptors must be activated by phosphorylation from associated active kinases.

Upon ligand binding Otk may be activated by phosphorylation catalyzed by an associated kinase in a similar manner to the erythroblastosis B (ErbB) 3 dead kinase. The epidermal growth factor receptor (EGFR) family member ErbB3 has impaired kinase activity, but can heterodimerize with kinase-active EGFR or ErbB2 to become activated by phosphorylation and transduce signals downstream (Kim *et al.*, 1998). Identification of the active tyrosine kinase that can phosphorylate Otk will be required to understand Otk signaling in R-cell growth cones. As well, the upstream ligand of Otk in R-cell

axons is unknown. While Sema1a functions as the upstream Otk ligand of Otk in motor axons (Winberg *et al.*, 2001), Sema1a does not appear to be the Otk ligand involved in R-cell axon targeting.

4.3 Sema1a does not appear to be an Otk ligand in the visual system

Winberg *et al.* (2001) demonstrated in the developing motor nervous system that *sema1a*, *plexA*, and *otk* have similar LOF phenotypes and that Otk and PlexA are associated in co-transfected COS cells. As Sema1a is thought to be a PlexA ligand (Winberg *et al.*, 1998), these genetic and biochemical results suggest that Sema1a acts upstream from Otk and PlexA in the guidance of motor axons. Our data presented in Chapters 2 and 3 suggest that Sema1a and Otk have individually separate functions in the *Drosophila* visual system for the following three reasons:

First, if Sema1a acts as an upstream activating ligand from both PlexA and Otk, we would predict Sema1a to be expressed in the lamina layer where Otk-expressing R-cell axons, once reaching the lamina, would be triggered to defasciculate from the R8 pioneer to terminate correctly. Contrary to this model, we found *sema1a* to be expressed in both R-cell axons and optic lobe neurons. If the role of Sema1a in the visual system were to trigger a layer-specific halt in R-cell outgrowth via PlexA and Otk, Sema1a would be unlikely to be expressed along the tracts of Otk-expressing R-cells.

Second, in the visual system *sema1a* and *otk* have different LOF phenotypes. While *otk* mutants exhibited a layer-specific targeting defect of R1-R6 axons, these axons extended toward the optic lobe in a proper, retinotopic fashion. Only in *sema1a* mutants have we observed defects in R-cell retinotopic organization. If Sema1a acts as an upstream activating ligand of Otk, we would predict similar LOF phenotypes for both

sema1a and *otk*, as observed in the developing motor nervous system (Yu *et al.*, 1998; Winberg *et al.*, 2001).

Finally, as Otk is required for the proper layer-specific termination of R1-R6 axons in the lamina, we would predict that overexpression of the Otk-ligand in the eye would lead to the early termination of R-cell axons. An early termination phenotype of R-cell axons was not observed when Sema1a was overexpressed in the eye. Alternatively, we found that *sema1a* overexpression led to R-cell hyper-fasciculation, with no early termination of R-cell axons.

4.4 Sema1a is required to establish the proper topographic termination pattern in the optic lobe

While Sema proteins have traditionally been described as repulsive guidance molecules (Mark *et al.*, 1997; Yu and Kolodkin, 1999; Tamagnone and Comoglio, 2000), a growing body of evidence has emerged that describe alternative functions for Sema molecules. For example, the vertebrate Sema-family member Sema3A has been demonstrated as capable of simultaneously attracting apical dendrites while repelling cortical axon growth (Polleux *et al.*, 2000). The attractive or repulsive effect of Sema3A on cultured *Xenopus* spinal neurons can be altered by changes in soluble Guanylate Cyclase levels (Song *et al.*, 1998) demonstrating that growth cone responses to Sema-signaling may be converted by a change in cyclic nucleotide levels. Additionally, the ectopic expression of *sema1a* has been shown to attract growth of the grasshopper Ti1 axons (Wong *et al.*, 1997). The observations that Sema proteins may act in an attractive manner and that axonal response to Sema proteins may be converted by altering levels of cyclic nucleotides indicate that axonal response to Sema-signaling is not limited to a repulsive action. Our results described in chapter 3 suggest yet another alternative

function for Sema1a in nervous system development: the promotion of R-cell axon topographic map formation in the optic lobe by promoting the attractive interaction of neighboring R-cell growth cones.

In *sema1a* mutant third-instar larva, although initial R-cell axon outgrowth appeared normal, we observed greatly disorganized lamina layers. We also noted *sema1a* mutant axons that completely ignored the normal retinotopic arrangement of R-cell axons in the optic lobe and crossed the paths of numerous neighbouring axons. These results are consistent with a requirement of Sema1a for maintenance of the proper organization of R-cell axonal growth, possibly by mediating the attractive interaction between neighboring growth cones. When approaching the lamina target region, R-cell growth cones interact in a stereotyped manner, possibly to provide guidance information for topographic pattern formation (Meinertzhagen and Hanson, 1993). According to this model, loss-of-*sema1a* would result in an inability of R-cell growth cones to be attracted to one-another resulting in random axonal extension toward the lamina, axons that cross the paths of neighbouring axons, and ultimately, the formation of a loosely associated lamina layer.

To test the hypothesis that Sema1a mediates an attractive interaction of R-cell axon growth cones, we overexpressed Sema1a in the eye. Consistent with our hypothesis, we observed a severe, dosage-dependant *sema1a* GOF phenotype consisting of R-cell axon hyper-fasciculation. An alternative explanation for the *sema1a* GOF phenotype is the possibility that the increased expression of Sema1a in R-cells leads to greater repulsion among R-cells and their lamina target cells. However, due to the observation that the *sema1a* LOF phenotype resulting in a loosely associated lamina layer

is autonomous to R-cell axons, we favour the hypothesis that increased Sema1a expression in the eye leads to an increase in R-cell axon attraction.

The *sema1a* hyper-fasciculation GOF phenotype was similar to the GOF phenotype observed when the cell-adhesion molecule Fas II was overexpressed in the eye. As well, we found that co-overexpression of both Fas II and Sema1a led to an enhancement of the Sema1a GOF phenotype. This result suggests that Sema1a and Fas II act in similar manners to promote the attraction of axons. If Sema1a behaves as a repulsive trigger for axonal defasciculation, we would predict that the overexpression of the cell-adhesion molecule, Fas II would antagonize the Sema1a GOF phenotype—a relationship that had previously been described for Fas II and Sema1a in the motor nervous system (Yu *et al.*, 2000). Together, these results indicate that the role of Sema1a in visual system development differs from the role described for Sema1a in the guidance of motor axons, where in the visual system Sema1a acts in an attractive manner to establish the proper topographic termination pattern in the optic lobe.

4.5 Sema1a acts as a receptor in R-cell axons

While Yu *et al.* (1998) have demonstrated the expression of the Sema1a extracellular domain is sufficient to rescue the *sema1a* mutant phenotype in the developing motor nervous system, indicating a role for Sema1a as a ligand, we made two observations that support the idea that Sema1a acts as a receptor to promote R-cell axon fasciculation. First, we found that only the expression of full-length Sema1a, but not a truncated form of Sema1a lacking the cytoplasmic domain ($Sema^{\Delta cyt}$), could rescue the *sema1a* LOF phenotype. Second, we observed that expression of $Sema^{\Delta cyt}$ could not generate the *sema1a* GOF phenotype in a *wt* background. Additionally, the expression of

Sema$^{\Delta cyt}$ in *wt* individuals resulted in a phenotype that is similar, if not identical, to the *sema1a* LOF phenotype. This result suggests that Sema$^{\Delta cyt}$ is capable of binding to normal Sema1a-binding partners, preventing the interaction of these partners with the endogenous Sema1a protein resulting in a dominant-negative effect.

Previous studies have also demonstrated a role for Sema1a acting as a receptor. For example, the Sema1a cytoplasmic domain has been demonstrated as necessary for complete rescue of *sema1a* defects in formation of the *Drosophila* giant synapse and to generate a GOF phenotype in the giant fiber (Godenschwege *et al.*, 2002). Similarly, the expression of the PlexA1 extracellular domain alone is sufficient to rescue a *plexA1* LOF phenotype in embryonic ventricular development of the chick, but not a *sema6d* LOF phenotype (Toyofuku *et al.*, 2004). The result of Toyofuku *et al.* (2004) suggests that chick ventricular development requires PlexA1 as a ligand for Sema-signaling.

4.6 Does PlexA act as a Sema1a ligand in R-cell axons?

To test the potential role of PlexA acting as a Sema1a ligand in the visual system, as has been demonstrated in the chick by Toyofuku *et al.* (2004), we completely removed *mical*, a downstream target of *plexA,* from the *sema1a* GOF background. We found that removal of *mical* in the *sema1a* GOF background did not suppress the *sema1a* GOF phenotype, supporting the idea that Sema1a-signaling in R-cells may not require PlexA to act as a receptor. Further work will be required to determine if PlexA acts as a Sema1a ligand in the visual system. The experiments necessary to test the possibility that PlexA is a Sema1a ligand in the visual system are described in the following section.

4.7 Future work

The upstream ligands and downstream targets necessary for Otk and Sema1a signaling in R-cells are currently unknown, however the identification and characterization of these molecules would yield important insight into the molecular mechanisms of Otk and Sema1a function. In this section I will describe experiments that would be used to identify these molecules.

One potential method for the identification of the downstream components in the Otk pathway that link the receptor to the underlying cytoskeletal elements would be the use of the Otk cytoplasmic domain as a "bait" molecule in a Y 2-H *Drosophila* cDNA library screen. Downstream molecules that physically interact with the DInR (Song *et al.*, 2003) and PlexA receptors (Terman *et al.*, 2002; Terman and Kolodkin, 2004) have been identified using a similar strategy. One disadvantage of using a biochemical screen to identify potential Otk-interacting molecules is the possibility of identifying "false positive" proteins that only interact with Otk *in vitro* but are not true components of the Otk pathway in R-cell growth cones. For this reason, candidate genes identified as positive in the Y 2-H screen would be analyzed for expression in R-cells, the LOF phenotypes of these genes would be examined, and the dosage-dependant interaction with *otk* in a sensitive *otk* genetic background of candidate genes would be tested. The removal of 50% gene dosage of an *otk*-interacting gene, where Otk normally activates the protein encoded by the *otk*-interacting gene would be predicted to enhance a sensitive *otk* phenotype. By using the combination of a biochemical approach to screen for Otk-interacting candidate molecules, testing candidate gene expression and LOF phenotypes, and examining the potential genetic interaction of these genes with *otk in vivo*, the

downstream molecules necessary for the function of Otk in lamina-specific targeting of R1-R6 growth cones can be identified. Similarly, the use of the Otk extracellular domain as a "bait" molecule in a Y 2-H screen of a *Drosophila* cDNA library could potentially identify an Otk ligand. The expression pattern, LOF phenotype, and potential genetic interaction with Otk of any positive candidates would be necessary to test the validity of an *in vitro* physical interaction.

The downstream molecules required for Sema1a signaling to the growth cone are unknown. In order to identify potential downstream Sema1a-interacting candidate genes, a screen for modifiers of the *sema1a* GOF phenotype may be undertaken. A similar strategy was used by Ruan *et al.* (2002) to identify Bif, the downstream target of Msn involved in R-cell targeting. A screen for *sema1a* GOF phenotype-modifiers could include crossing P-element insertions into flies that overexpress Sema1a in the eye and examining the resulting phenotype. Dosage reduction by 50% of a P-element disrupted gene that is normally activated by Sema1a downstream would be hypothesized to decrease Sema1a signaling and suppress the *sema1a* GOF phenotype. Sema1a-interacting genes would next be examined for expression in R-cells, and the LOF phenotypes of these genes would be characterized to examine for similarity with the *sema1a* LOF phenotype. Finally, direct physical interaction of Sema1a and potential Sema1a-interacting proteins could be tested by a variety of biochemical experiments, including GST-fusion protein pull-down and Y 2-H binding assays. The approach of identifying modifiers of the *sema1a* GOF phenotype, assessing the functions of *sema1a*-interacting genes *in vivo*, and examining the physical interaction of Sema1a and Sema1a-

interacting candidate proteins *in vitro* would reveal the downstream components of Sema1a signaling in R-cells.

While the Sema1a ligand in R-cells is unknown, Toyofuku *et al.* (2004) have demonstrated that PlexA1 acts as a ligand for the vertebrate Sema6d, suggesting the potential for PlexA to act as a Sema1a ligand. In order to test the hypothesis that PlexA is a Sema1a ligand in R-cell axon fasciculation, a truncated form of PlexA lacking the cytoplasmic domain ($PlexA^{\Delta cyt}$) would be expressed in the eye of *wt* individuals. If the eye-specific expression of $PlexA^{\Delta cyt}$ in a *wt* background appears similar to the *sema1a* GOF phenotype, then PlexA may activate the Sema1a signaling pathway as a ligand. If eye-specific $PlexA^{\Delta cyt}$ expression caused a LOF phenotype—that is, if $PlexA^{\Delta cyt}$ behaves in a dominant-negative manner, then signaling may also occur through PlexA acting as a receptor. Finally, the lack of a change in phenotype resulting from $PlexA^{\Delta cyt}$ expression in the eye may indicate a lack of PlexA involvement in Sema1a signaling in R-cells. As well, to determine if endogenous PlexA is required for the proper establishment of R-cell projection patterns, RNAi can be used to knock down PlexA expression in the eye with an eye-specific driver. The effect of removing PlexA expression from specific R-cell subtypes can be accomplished by the expression of RNAi constructs using a variety of R-cell subtype-specific drivers. These experiments to test for PlexA involvement in Sema1a signaling are currently underway in our laboratory.

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Appendix 1.

Detailed methods

A.1 Dissection and staining of imaginal tissue and adult brains

Eye-brain complexes were dissected from third-instar larva and brains were dissected from adult heads in ice cold phosphate-buffered saline (PBS) (adjusted to pH 7.0) and fixed for 50 minutes at room temperature in PLP (2% formaldehyde, 0.075 M lysine, 0.1 M sodium phosphate buffer, pH = 7.4). The tissue was washed 3 times for 10 minutes each in PBT (0.5% Triton X-100 in PBS) and blocked for one hour with 10% normal goat serum (NGS) in PBT. R-cells were stained with primary antibodies (AB) as described in the section A.3 below, diluted in 10% NGS/PBT overnight at 4°C. The samples were again washed 3 times for 10 minutes each in PBT and incubated with goat anti-mouse or goat anti-rabbit secondary AB to recognize either monoclonal or polyclonal primary AB, respectively. The secondary AB were diluted 1 to 200 in 10% NGS/PBT. Prior to development in 3,3'-diaminobenzidene (DAB) staining solution (0.5 mg/ml DAB, 0.003% hydrogen peroxide (H_2O_2)) when using horseradish peroxidase conjugated secondary AB, or exposure to ultraviolet light when using Texas Red or fluorescein (FITC) conjugated secondary AB, the tissue was washed once for 10 minutes in PBT, and 3 times 10 minutes in PBS. Tissue was washed a final 3 times in PBS prior to mounting in 70% glycerol in PBS.

A.2 Cryostat sectioning and staining of frozen heads

Proboscises were removed from decapitated adult heads that were fixed in 4% formaldehyde diluted in PBS for 4 hours at 4°C. The heads were washed twice for 15 minutes each in PBS and dehydrated by submersion in 25% sucrose/PBS overnight at

4°C. The heads were embedded in Tissue-Tek optimal cutting temperature (OCT) compound (4583) within a cryomold (Tissue-Tek, 4565) and frozen by immersion of the molds in 2-methylbutane (Aldrich, 27, 034-2) and placing the samples/2-methylbutane in a liquid nitrogen bath. The heads were cut in 10 μm sections at -20°C using a Leica CM3050 S cryostat and collected on glass slides coated with 0.01% poly-L-lysine (Sigma, P 8920). The sectioned heads were blocked for 30 minutes at room temperature with blocking solution (10% NGS, 1X PBS, and 0.1% Triton X-100) and R-cells were stained with primary AB as described in the section A.3 below, diluted in 10% NGS/PBS overnight at room temperature. Samples were washed twice for 15 minutes each in PBS and incubated in the appropriate anti-mouse or anti-rabbit secondary antibody diluted in 10% NGS/PBS. Samples were washed 3 times 15 minutes each in PBS and either developed in DAB staining solution or exposed to UV light.

A.3 Antibody staining of tissue

R1-R8 axons were visualized in third-instar larva and cryostat-sectioned adult heads using the mAB 24B10 (1 to 100 dilution, Developmental studies hybridoma bank (DSHB)) (Fujita, 1982), an antibody that recognizes the Chaoptin protein expressed in all R-cells (Reinke, 1988; Krantz, 1990). The R-cells of hemispheres labeled with *rough-τ-lacZ* (R2-R5) (Heberlein, 1990; Callahan, 1994), *Rh1-lacZ* (R1-R6) (Mismer and Rubin, 1987), *glass-lacZ* (R1-R8) (Mismer and Rubin, 1987), or *UAS-lacZ* were stained with an anti-β-galactosidase AB (1 to 100 dilution). Third instar eye-imaginal discs were also stained with anti-Repo (1 to 10 dilution, DSHB), anti-Bride-of-sevenless (Boss) (1 to 2000 dilution, DSHB) (Reinke, 1988), and anti-Prospero (1 to 200 dilution, DSHB) (Kauffmann, 1996) AB to visualize glial cells, R8, and R7 and cone cells, respectively.

Cobalt chloride ($CoCl_2$) was added to a 1.5 mM final working concentration in DAB during anti-Prospero analysis of R7 in order to intensify the reaction product. The R7 projection patterns of adults carrying the PANR7-GAL4::UAS-Synaptobrevin-GFP marker were stained with an anti-Green fluorescence protein (GFP) AB (1 to 1000 dilution, Molecular Probes). The expression pattern of Otk was assessed by double staining third-instar eye-brain complexes with both 24B10 and an anti-Otk AB (1 to 100 dilution) (Pulido *et al.*, 1992). The expression pattern of Sema1a was assessed by double staining third-instar eye-brain complexes with both 24B10 and an anti-Sema AB (1 to 100 dilution, gift from Dr. Alex Kolodkin).

A.4 Quantification of axonal bundles in the medulla in *otk* and *sema* mutants

The percentage of mistargeted R2-R5 axon bundles that passed through the lamina and entered the medulla of *otk* and *sema* mutants was estimated following the method described by Garrity *et al.* (1999). This method involved dividing the average number of mistargeted R2-R5 axons in the medulla from the total average number of R2-R5 bundles estimated as contacting the medulla. Garriety *et al.* (1999) subtracted the R2-R5 axons of 4-5 ommatidial rows from their estimate of the total number of R2-R5 bundles, presuming that the axons from these rows had not reached the brain. In our study, due to the younger average age of examined individuals, a greater number of ommatidial rows that sent axons into the brain were observed than reported by Garrity *et al.* (1999). This observation was taken into account in our calculations by not subtracting the 4-5 ommatidial rows from our mean number of ommatidial rows that Garrity *et al.* (1999) presumed had sent out axons that did not yet reach the brain.

A.5 Plastic sections of retinal tissue

The following steps to prepare retinal tissue for microtome sectioning were all performed on ice. Retinas were dissected from adult heads in PBS and fixed in 4% formaldehyde in PBS for 40 minutes. The retinas were transferred to 2% glutaraldehyde for 45 minutes for further fixation. The tissue was rinsed twice for 5 minutes each in PBS and dehydrated for 5 minute intervals each in: 50%; 70%; 80%; 95%; 100%; 100%; and 100% ethanol.

The samples were equilibrated in propylene oxide for 2 times 5 minutes each and transferred to 1:1 propylene oxide:resin at room temperature before placing the tissue in 100% resin to embed in a rubber mold. The resin solidified when baked overnight in a 60°C oven. The retinas were cut in 1.5 μm sections using a Reichert-Jung Ultracut E microtome, picked up on glass slides, and stained with toluidine blue staining solution (1% toluidine blue and 1% borax) on a 75 °C heating block for approximately 30 seconds, and rinsed with water.

Appendix 2.

Table 2: Molecules involved in R-cell axon guidance and targeting

Guidance molecule	Classification	Effect on R-cell axons
Brakeless	Nuclear protein that represses Runt expression in R2 and R5 R-cells	Required for R1-R6 lamina-specific targeting
Runt	Nuclear protein	Required for R1-R6 lamina-specific targeting
Flamingo	Cadherin-related cell surface protein	Required for R1-R6 axons to form proper connections with target lamina neurons and for R8 axon targeting
PTP69D	Receptor tyrosine phosphatase	Required for R1-R6 lamina-specific targeting and R7 layer termination
LAR	Receptor tyrosine phosphatase	Required for R1-R6 axons to form proper connections with target lamina neurons and for R7 axon targeting
N-Cadherin	Calcium-dependent cell adhesion molecule	Required for R1-R6 axons to form proper connections with target lamina neurons and for R7 axon targeting
DInR	Receptor tyrosine kinase	Required for R-cell axon guidance
Otk	Receptor tyrosine kinase	Required for R1-R6 lamina-specific termination
Dock	SH2 and SH3 domain-containing adaptor molecule	Required for R1-R6 lamina-specific termination and R-cell axonal guidance
Misshapen	Serine/threonine kinase	Required for R1-R6 lamina-specific termination
Bifocal	Regulator of the cytoskeleton	Required for R1-R6 lamina-specific termination
Pak	Serine/threonine kinase	Required for R-cell axonal guidance
Trio	Guanine nucleotide exchange factor	Required for R-cell axonal guidance